Relationship between Parkin Function, Heavy Metal Homeostasis and Oxidative Stress in a *Drosophila* Model of Parkinson's Disease

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Dedicated to my Parents

Rajendra Kumar Saini

and

Kanchan Saini

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ZUSAMMENFASSUNG

Die Parkinsonsche Krankheit ist die zweithäufigste neurodegenerative Erkrankung. Die Haupt-Symptome sind Tremor (unkontrolliertes Zittern) und maskenhafte Gesichtszüge. Auf zellulärer Ebene geht die Krankheit einher mit fortschreitendem Verlust von Neuronen der *Substantia nigra* im Zentralnervensystem. Die meisten Fälle sind idiopathisch, d.h. ohne erkennbaren Auslöser, doch kommt die Krankheit auch familiär gehäuft vor, wobei Mutationen in bisher sechs Genen als Ursache identifiziert werden konnten. Eine Untergruppe der autosomal-rezessiven form der Parkinsonkrankheit wird durch Mutation im Parkin-Gen, das für eine E3 Ubiquitinligase kodiert, bewirkt. Es gibt mehrere Hinweise darauf, dass oxidativer Stress die Parkinsonkrankheit begünstigt. Weil eine Störung im Schwermetallhaushalt der Zelle oxidativen Stress bewirken kann, haben wir bei einer *Parkin*-Mutante der Essigfliege *Drosophila melanogaster* den Effekt einer veränderten Schwermetall-Homöostase getestet. Homozygote *Parkin*-mutante Fliegen zeigen schwere motorische Defekte einschliesslich Flugunfähigkeit, männliche und weibliche Sterilität, und stark verkürzte Lebensdauer. Wir konnten zeigen, dass eine Doppelmutante für *Parkin* und MTF-1 (metal responsive transcription factor, auch metal response element binding transcription factor genannt) nicht lebensfähig ist, d.h. “synthetische Letalität” zeigt. Die hauptsächlichen Zielgene von MTF-1 kodieren für Metallothioneine, kleine cysteinhaltige Proteine welche in der Lage sind etliche Metalle, z.B. Kupfer, Zink, Kadmium und Quecksilber zu binden, oder Radikale zu neutralisieren. Interessanterweise zeigen transgene Fliegen mit erhöhtem MTF-1-Spiegel eine dramatische Verlängerung der Lebensdauer und eine verbesserte Motorik, welche sogar zu kurzen Flügen befähigt. Zudem sind die Weibchen (allerdings nicht die

Männchen) fruchtbar. Auf zellulärer Ebene ist die Struktur der Ovarien und der Flugmuskeln weitgehend normalisiert. Weitere Experimente mit Parkin-Mutanten ergaben starke Verbesserungen im Phänotyp auf Futter mit BPS und BCS, zwei Chelatoren mit Präferenz für die redox-aktiven Metalle Eisen und/oder Kupfer. Schliesslich wurden auch noch eine verlängerte Lebensspanne, verbesserte Motorik und teilweise Wiedergewinnung der weiblichen Fertilität beobachtet, wenn *Parkin*-Mutanten auf Futter mit Zink-Zusatz aufgezogen und gehalten wurden. Ein besonders günstiger Effekt wurde dabei mit einer hohen Konzentration (4 mM Zinkchlorid) beobachtet, welche bei Kontroll-Fliegen bereits Vermeidungsreaktionen und einen verschlechterten Gesamtzustand bewirkten. Zink ist, im Gegensatz zu Kupfer und Eisen, kein redox-aktives Metall und wirkt unter den meisten Bedingungen als Antioxidans, was zumindest teilweise den positiven Effekt auf *Parkin*-Mutanten erklären könnte. Zusammengefasst lässt sich sagen, dass unsere Studien eine Verbindung zwischen Parkin-Funktion und Schwermetall-Homöostase nahelegen, welche auch für Säuger einschliesslich des Menschen von Bedeutung sein könnte.

SUMMARY

Parkinson's disease (PD) is the second most common neurodegenerative disease. The symptoms are tremor and rigidity and a mask-like face expression. At the cellular level PD is caused by progressive loss of neurons in a brain region termed *Substantia nigra*. PD in most cases is idiopathic, but a fraction is caused by specific dominant or recessive mutations in either of six genes known. Some autosomal recessive PD are caused by mutations in the gene for Parkin, a E3 ubiquitin ligase. Several lines of evidence point to oxidative stress as a major cause of PD. Since a distortion of cellular heavy metal homeostasis has been associated with increased oxidative stress, we analyzed the response of a *parkin* mutation ($park^{25}$) in the vinegar fly *Drosophila melanogaster* to modulation of heavy metal homeostasis. *Drosophila* homozygous *parkin* mutants suffer from severe motoric defects including inability to fly, male and female sterility, and a very short life span. We found that a combination of a *parkin* mutation with a loss-of-function mutation of MTF-1 (metal responsive transcription factor, also referred to as metal response element binding transcription factor) is not viable, i.e., displays a "synthetic lethal phenotype". The major target genes of MTF-1 are encoding metallothioneins, small cysteine-rich proteins with the ability to bind a number of metals, including copper, zinc, cadmium and mercury, and also to scavenge radicals. Interestingly, transgenic flies with elevated MTF-1 expression show a dramatic prolongation of life span and improvement of motoric abilities which even includes short patches of flight. Furthermore the females (but not the males) are fertile. At the cellular level, muscle and ovary morphology are largely normalized. In an independent set of experiments with *parkin* mutants, we also find a beneficial effect upon food supplementation with BPS and BCS,

chelators with a preference for the redox-active metals iron and/or copper. Finally, improved life span, motoric abilites and partial recovery of female fertility is also observed when *parkin* mutants are raised and kept on food with a supplement of zinc ions. A particularly good effect is seen at a food zinc concentration (4 mM) which is already showing avoidance reactions and adverse effects in control flies. Zinc, unlike iron and copper, is not redox-active and under most circumstances acts as an anti-oxidant, which might at least in part explain its positive effect on the *parkin* mutant flies. Taken together, our findings point to an intriguing interplay between Parkin function and metal homeostasis which may well have relevance to mammals including humans.

INTRODUCTION

I Parkinson's disease
II MTF-1
III zinc homeostasis

I)Parkinson's disease

1.1 Clinical and pathological features of Parkinson's Disease

Parkinson's disease (PD) is the most common chronic movement disorder and the second most common neurodegenerative disorder which was first described by James Parkinson in 1817. The incidence of this disease, markedly increases with age (Twelves et al., 2003), typically manifesting itself after the age of 50, with an estimation of about four million people worldwide and 1% of the population above the age of 65 years being inflicted (Bushnell and Martin, 1999; Chung et al., 2003). Clinical manifestations of PD patients include a broad range of motor abnormalities including rigidity, bradykinesia (slowness of voluntary movement), resting tremor and postural instability. The movement disorder in PD can be largely credited to the selective loss of dopaminergic neurons in the nigrostriatal pathway of the brain (Dawson, 2000; Parkinson, 2002; Spacey and Wood, 1999). There are several secondary symptoms, such as constipation, difficulties in swallowing, loss of bladder and/or bowel control, abnormalities in olfactory and visual perception, bradyphrenia (slow response to questions) and hypophonia (soft voice) in addition to psychosocial disorders, such as, anxiety and depression as well as dementia and cognitive impairment (Gelb et al., 1999). In order to be surely diagnosed with PD, a patient must have at least two or more of the primary symptoms, one of which should be either resting tremor or bradykinesia. A caveat is that several other diseases display similar symptoms defined for PD making a definitive diagnosis possible *post mortem*. The motoric impairments in PD are usually responsive to levodopa (L-DOPA), resulting in the improvement of these clinical symptoms (Gelb et al., 1999).

Pathologically, PD is characterized by the presence of cellular inclusions called "Lewy bodies" and a progressive but selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) which is necessary for normal motor function.

This selective loss of dopaminergic neurons ultimately leads to the loss of dopamine (DA), its metabolites, 3,4 dihydroxyphenylacetate (DOPAC) and homovanillic acid (HVA), the dopamine transporter (DAT) in the striatum and the SNc, and its biosynthetic enzyme, tyrosine hydroxylase (Zhang et al., 2000). The first PD symptoms develop when about 70% of the striatal dopamine neurons and about 50% of the nigral dopamine neurons are lost (Dunnett and Bjorklund, 1999; Lansbury and Brice, 2002). Friedrich Lewy first described the pathological hallmark of PD, Lewy bodies in 1912 as a hyaline inclusion in the neuronal cytoplasm. Classical Lewy bodies are 5-25 μM fibrillar, proteinaceous cytoplasmic inclusions with a dense eosinophilic core surrounded by a clear halo and are usually found in the SNc and locus coeruleus of PD patients (Huang et al., 2004; Pollanen et al., 1993). Misfolded proteins can be generated due to genetic mutations (Goldberg et al., 2003) or oxidative modifications (Dalle-Donne et al., 2006) and accumulate in Lewy bodies, most likely due to a chronic imbalance in their generation and clearance. The main component of Lewy bodies is α-Synuclein (Goedert and Spillantini, 1998; Iwatsubo, 2003; Spillantini et al., 1997). In some Lewy bodies, several other proteins were shown to be present besides α-Synuclein. These include Ubiquitin (Manetto et al., 1988), Ubiquitin-carboxyterminal hydrolase (UCH-L1) (Lowe et al., 1990), Synphilin-1 (Wakabayashi et al., 2000), Tau (Ishizawa et al., 2003), Parkin-associated-endothelin receptor-like receptor (Pael-R) (Murakami et al., 2004), Parkin (Schlossmacher et al., 2002), neurofilaments (Schmidt et al., 1991), Torsin A (Shashidharan et al., 2000) and heat shock proteins such as Hsp70 and Hsp90, which act as molecular chaperones (Auluck and Bonini, 2002). Misfolded proteins are prone to aggregate formation and have the potential to impair cellular functions (Gregersen et al., 2006). Lewy bodies most likely have a protective function by sequestering these potentially cytotoxic proteins and preventing them from damaging cellular organelles present in the cytoplasm. In agreement with this idea, dopamine neurons containing Lewy bodies have been reported to escape from degeneration in PD, suggesting that Lewy-body positive neurons are less vulnerable than their Lewy-body negative counterparts (Tompkins and Hill, 1997).

Neurobiochemical implications of PD include mitochondrial complex I dysfunction, reduced glutathione, increased lipid peroxide and iron levels and decreased ferritin

concentrations which suggest that dopaminergic neurons of PD patients are under oxidative stress (Kosel et al., 1999; Munch et al., 1998). Furthermore, recent studies have implied altered proteasomal function as one of the contributing factors in the pathogenesis of PD (McNaught et al., 2003).

Evidence suggests that both environmental and genetic factors play contributing roles in the etiology of the disease. Both exogenous environmental toxins and endogenous proteotoxins have been implicated in PD pathogenesis (Yang et al., 2003). The molecular mechanism(s) underlying the selective dopaminergic neuronal loss in PD are not clearly outlined, but accumulating evidence indicate that mitochondrial dysfunction, oxidative stress, impaired ubiquitin-dependent proteolysis and protein aggregation are involved in the pathogenesis of PD. Mutations in a number of genes have been found to be linked to various inherited forms (familial) PD and several of them are associated with the ubiquitin-proteasome protein degradation pathway. Although inherited forms of PD account for only about 5-15% of known PD cases (de Silva et al., 2000; Gasser, 2001; Mizuno et al., 2001), the symptoms and neuropathology are essentially identical to idiopathic PD. However, most PD cases are still believed to be sporadic. Hence, the hunt for the possible environmental contaminants or other non-genetic causes that are linked to the development of PD still remains an active area of research.

1.2 Genetics of PD

1.2.1 Parkin (PARK2)

The gene *PARK2* also known as *parkin* was identified by positional cloning in Japanese families (Kitada et al., 1998). The locus *PARK2* responsible for causing autosomal-recessive juvenile PD (AR-JP), characterized by early-onset, slow disease progression and mostly absence of Lewy bodies, barring an exceptional case (Farrer et al., 2001; Mori et al., 1998; Takahashi et al., 1994) was mapped to chromosome 6q25.2-27 (Matsumine, 1999).

The *parkin* gene is one of the largest genes in the human genome (1.53 Mb), containing 12 exons and encoding a 465-amino acid protein of 52 kDa molecular weight. The *parkin* promoter functions as a bi-directional promoter. It regulates the

transcription of *parkin* on the one hand and a gene upstream of *parkin* in an antisense orientation, called *PACRG* which encodes Parkin co-regulated gene (PACRG) or Gene located upstream of *parkin* (Glup) on the other hand (West et al., 2003).

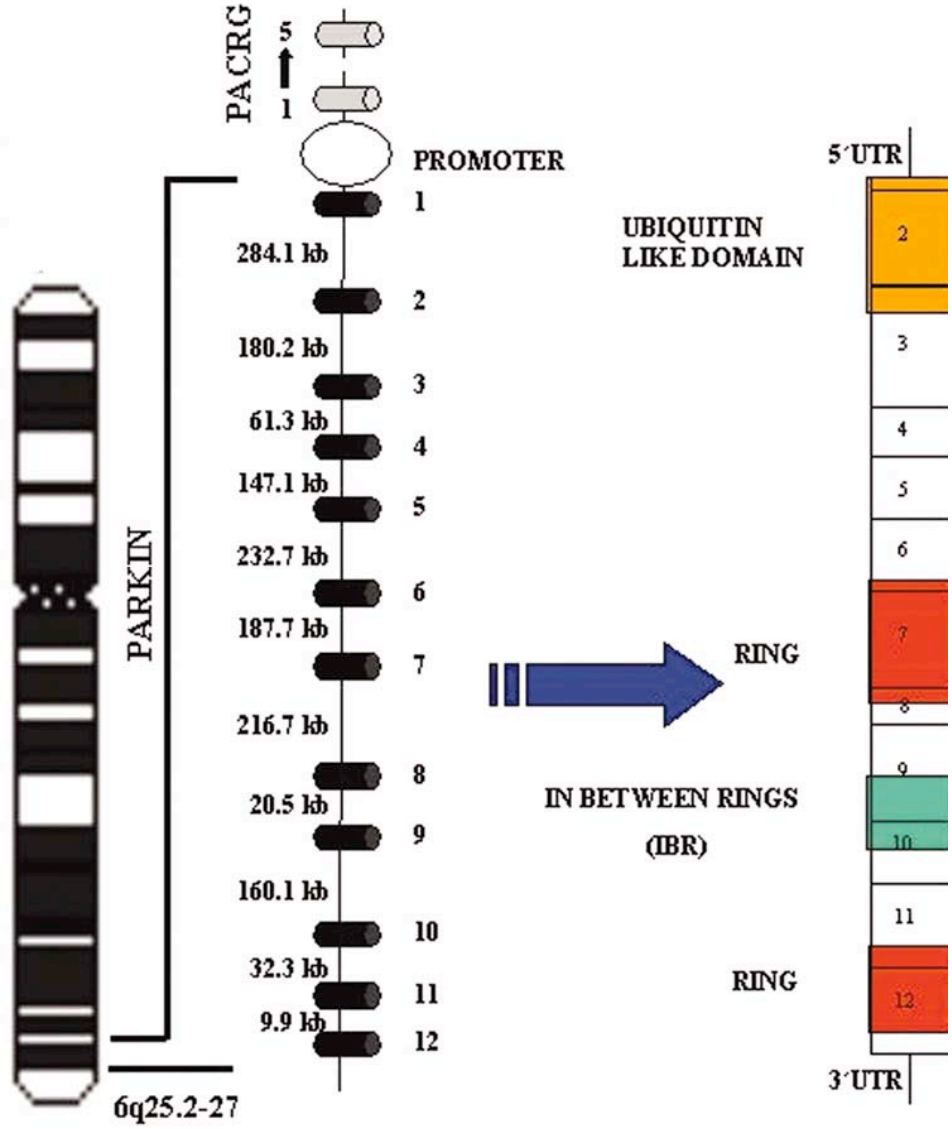

Figure 1. Schematic representation of the Parkin/PACRG locus.
The chromosomal location is shown (left). 12 individual exons of *parkin* are marked (not to scale) with intron sizes indicated (middle). Protein domain structure is indicated (right). Adapted from Mata et al., 2004.

PACRG is a component of Lewy bodies and forms a part of the chaperone complex with Hsp70 and Hsp90 and it suppresses unfolded Pael receptor-induced neuronal cell death (Imai et al., 2003), however, its exact function is unclear at present. This suggests a model of toxicity reduction of Pael-R in dopamine neurons by the joint

action of Parkin which degrades its substrate, Pael-R and PACRG which sequesters Pael-R into Lewy bodies.

Structure, expression and function of Parkin

Besides a ubiquitous expression in the brain, Parkin is found in several cellular compartments, including the cytosol, trans-Golgi network, synaptic membrane compartment, postsynaptic densities, lipid rafts, cytoplasmic vesicles, endoplasmic reticulum, outer nuclear and outer mitochondrial membranes (Fallon et al., 2002; Kubo et al., 2001; Shimura et al., 1999; Stichel et al., 2000). Functional Parkin protein has been suggested to be a requirement for Lewy body formation as it has been found in Lewy bodies of autosomal dominant PD patients (Schlossmacher et al., 2002; Shimura et al., 2001). However, several other studies failed to detect Parkin in Lewy bodies or Lewy neurites by Parkin antibodies (Pawlyk et al., 2003).

Parkin has a ubiquitin-like (UBL) domain at its N-terminus and two RING (Really Interesting New Gene) fingers, a type of zinc finger flanking the IBR (In-Between-RING fingers) domain at its C-terminus. The UBL domain binds to the Rpn10 subunit of the 26S proteasome (Sakata et al., 2003) and is also involved in substrate binding (Shimura et al., 2000). The segment present in between the UBL and RING1-IBR-RING2 domains shows no clear similarity to known proteins and was accordingly termed the unique parkin domain (UPD) (Kahle et al., 2000a). The PDZ-binding motif at the C-terminus of Parkin interacts with the calcium/calmodulin-dependent serine protein kinase (CASK) (Fallon et al., 2002) which co-localizes with Parkin at postsynaptic terminals and in lipid rafts in rat brain *in vivo* as well as in synapses in cultured cortical neurons. There is high evolutionary conservation in Parkin, with orthologs in *Drosophila melanogaster*, *Caenorhabditis elegans*, mouse, rat and other species (Bae et al., 2003; Culetto and Sattelle, 2000). *Drosophila* Parkin shows 42% amino-acid sequence identity to human Parkin (Bae et al., 2003).

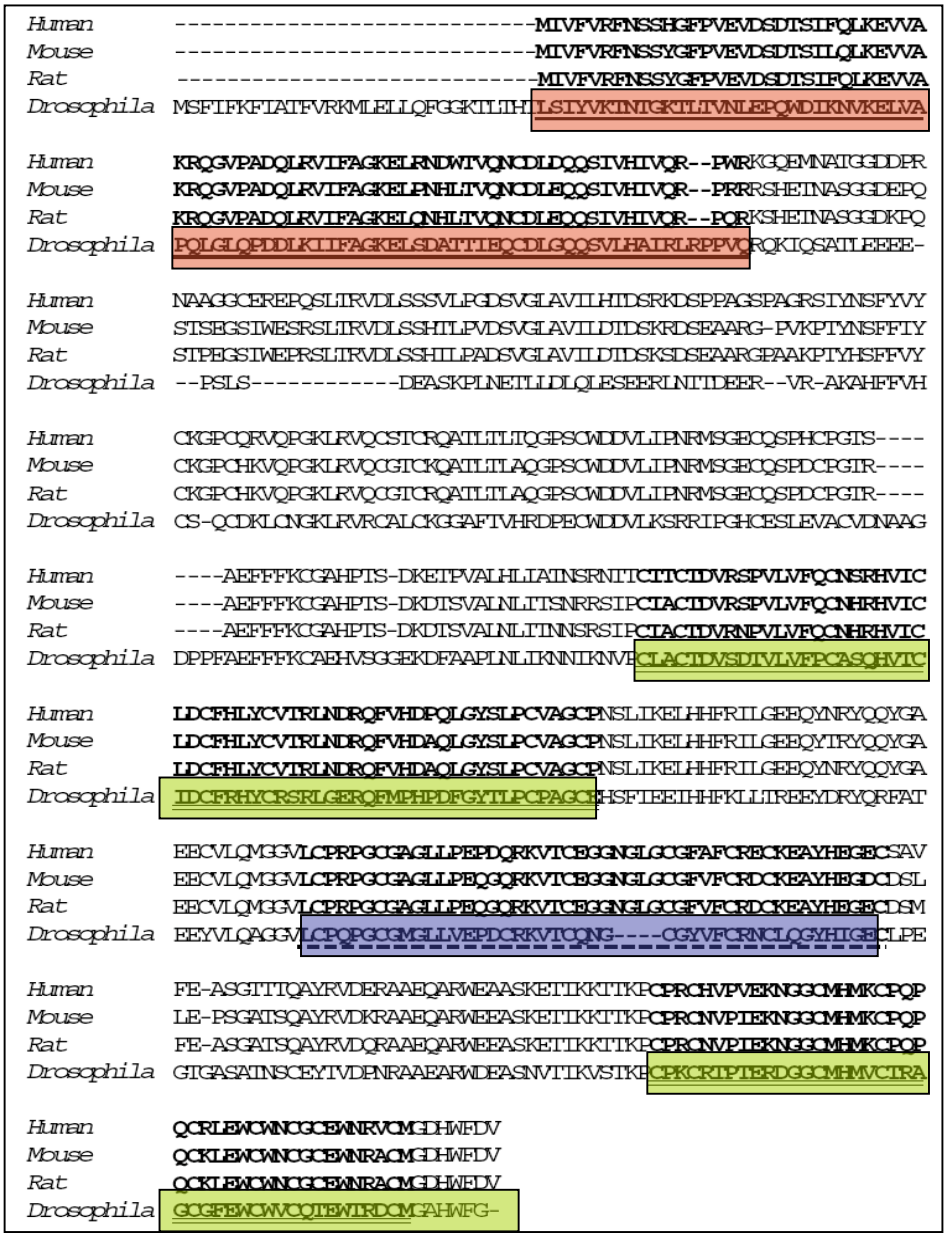

Figure 2: Alignment of the amino acid sequence of D. melanogaster parkin with human, mouse and rat. The conserved domains are in bold and ubiquitin-like domain is in red. RING domains are shown in green and IBR domain is indicated in blue. According to Bae et al., 2003.

Parkin functions as an E3 ubiquitin ligase

A number of RING finger proteins function as E3 ubiquitin protein ligase which play an important part in the ubiquitin proteasome system (UPS). The E3 ligases are responsible for the covalent attachment of Ubiquitin to proteins that are targeted for degradation by the proteasome (Joazeiro and Weissman, 2000). The UPS is involved in the temporal degradation of short-lived proteins which have important roles in cell-cycle, signal transduction and metabolism. Additionally, the UPS ensures protein quality control, it removes the misfolded or aggregated proteins by recruiting the E3

ubiquitin ligase, which further links the protein folding (chaperone) and degradation (proteasome) machinery of the cell.

Extensive molecular, genetic and functional analyses have shown that Parkin is an E3 ubiquitin protein ligase which is involved in protein turnover via the UPS (Shimura et al., 2000). Mutants for the Parkin RING domain lose their E3 ligase activity and the ability to bind to E2 ubiquitin conjugating enzymes, such as UbcH7 (Shimura et al., 2000). This indicates that the RING domain is required for recruitment of E2 enzymes into the Parkin complex. α-Sp22, a 22-kDa glycosylated form of alpha-synuclein notwithstanding, all Parkin substrates bind to Parkin via its RING finger domain (Shimura et al., 2001). Hence, mutations in the RING-IBR-RING domain can affect both substrate recognition as well as E2 binding, either of which results in partial or total loss of substrate degradation ability. Parkin can ubiquitinylate substrates by joining Ubiquitin moieties either via the classical lysine 48 (K48) (Pickart, 2001) or via the unclassical lysine 63 (K63), resulting in totally different outcomes, as displayed by the Parkin-binding protein Synphilin-1 which is an α-Synuclein-interacting protein (Lim et al., 2005). While K48-linked ubiquitination of Synphilin-1 promotes its proteasomal degradation, K63-linked ubiquitination by Parkin may be involved in the formation of Lewy body inclusions formed by the coexpression of α-Synuclein and Synphilin-1 associated with PD (Lim, et al. 2005) (Chung et al., 2001). The relative expression levels between Parkin and Synphilin-1 determine the Synphilin-1 degradation kinetics. Appreciable degradation of Synphilin-1 by Parkin occurs only at an unusually high Parkin to Synphilin-1 expression ratio or when the system is primed for lysine-48 (K48) ubiquitination. In contrast, parkin ubiquitinates Synphilin-1 via K63-linked ubiquitin chains when Synphilin-1 and Parkin are coexpressed at equivalent levels (Lim et al., 2005). This suggests that Parkin may act as a dual-function ubiquitin protein E3 ligase capable of mediating both proteasomal-linked (K48) and proteasomal-independent (K63) ubiquitination under different cellular conditions.

Mutations in *parkin* cause autosomal-recessive juvenile PD (AR-JP)

The most frequent form of familial PD is Parkinsonism due to *parkin* mutations. Half of the inherited PD cases are estimated to be caused by mutations in *parkin* (Lohmann

et al., 2003). (Huynh et al., 2003). Homozygous mutations usually result in the loss of Parkin's E3 ligase activity along with altered Parkin levels (Finney et al., 2003). Parkin mutations include exonic deletions, insertions and several missense mutations, with the hotspots for point mutations in the two RING finger motifs, the IBR as well as the UBL domain (von Coelln et al., 2004a). In addition to homozygous mutations, compound heterozygous mutations have been identified in patients that displayed PD symptoms clinically indistinguishable from idiopathic PD (Klein et al., 2000).

Substrates of Parkin

(A) Substrates forming components of Lewy bodies

A.1 α-Synuclein: Parkin interacts with a rare O-linked glycosylated form of α-Synuclein (α-Sp22) in Lewy bodies that were extracted from post-mortem brain tissue of AR-JP patients (Shimura et al., 2001), while mutant *parkin* fails to ubiquitinylate α-Sp22 *in vitro* (Chung et al., 2001). Parkin protects against α-Synuclein toxicity *in vitro* (Petrucelli et al., 2002) and prevents α-Synuclein induced dopaminergic neuron death in *Drosophila* and rat models of PD (Lo Bianco et al., 2004; Yang et al., 2003).

A.2 Synphilin-1

Synphilin-1 is a major component of Lewy bodies found in PD patients (Wakabayashi et al., 2000). Parkin ubiquitinates Synphilin-1, an α-Synuclein-interacting protein (Chung et al., 2001). Upon binding to synaptic vesicles Synphilin-1 possibly regulates the synaptic functions of α-Synuclein, by anchoring it to vesicle membranes (Ribeiro et al., 2002). Functional Parkin is essential for the formation of Lewy body inclusions. This is consistent with the absence of Lewy bodies in patients with AR-JP.

A.3 Pael-Receptor (Pael-R)

The Parkin-associated endothelin receptor-like receptor (Pael-R) is a putative G-protein-coupled multipass endoplasmic reticulum transmembrane protein which is abundantly expressed in dopamine neurons of the substantia nigra (Imai et al., 2001) and is found in the Lewy bodies of PD patients (Murakami et al., 2004). The folding of Pael-R is assisted by the 70-kDa heat-shock protein Hsp70. Overexpressed Pael-R

is insoluble and it triggers the so-called unfolded protein response which leads to ER stress. Insoluble Pael-R is ubiquitinated by Parkin with the help of ER-resident ubiquitin-conjugating enzymes (Ubc6 and Ubc7) and sent for proteasomal degradation before its accumulation (Imai et al., 2001).

A.4 p38 subunit of the aminoacyl-tRNA synthetase complex

The degradation of p38, a major structural component of the aminoacyl- tRNA synthetase complex is carried out by Parkin mediated ubiquitination (Corti et al., 2003). Overexpression of p38 induces cell death. p38 is found in Lewy bodies of patients with idiopathic PD.

A.5 Synaptotagmin XI

Synaptotagmin XI is localized to the core of Lewy bodies and its interaction with Parkin results in its ubiquitination and degradation (Huynh et al., 2003). The synaptotagmin family is crucial for vesicle formation and docking. The interaction with a member of the synaptotagmin family suggests an involvement of Parkin in the regulation of proteins involved in controlling neurotransmitter trafficking at the presynaptic terminal.

(B) Substrates regulating neurotransmitter secretion and uptake

B.1 Dopamine transporter (DAT)

DAT belongs to the catecholamine transporter protein family and is localized to the plasma membrane of dopamine neurons (Hersch et al., 1997). It regulates the uptake of dopamine from the synaptic cleft (Torres et al., 2003). Lack of glycosylation promotes the misfolding and aggregation of DAT which further oligomerizes with correctly folded DAT, affecting the targeting of normal DAT to the plasma membrane (Sorkina et al., 2003). Parkin significantly enhances the ubiquitination and subsequent degradation of misfolded DAT (Jiang et al., 2004). Results showing a significantly reduced DAT in the striatum of *parkin* knockout mice are in perfect agreement with the required role of Parkin in DAT targeting (Itier et al., 2003).

(C) Polyglutamine proteins

Polyglutamine proteins are aggregation-prone cytosolic proteins. Cytosolic protein-misfolding stress results in an upregulation of Hsp70 which further recruits Parkin

through its interaction with the RING-IBR-RING domain (Tsai 2003). The polyglutamine protein is then ubiquitinated and targeted for proteasomal degradation. This process might be facilitated by the direct binding of the UBL domain of Parkin to the regulatory 19S proteasome (Sakata et al., 2003).

(D) Substrates involved in cell cycle regulation and cytoskeleton structure

D.1 Septin family of proteins - CDCrel-1 and CDCrel-2a

Cell-division control-related protein-1 (CDCrel-1), also known as Sept5, belongs to the a family of GTPase called septin. It is predominantly expressed in the nervous system and is ubiquitinated and degraded by Parkin (Beites et al., 1999; Zhang et al., 2000). CDCrel-1 accumulates in the brains of AR-JP patients (Choi et al., 2003). CDCrel-2a, another member of the septin family having strong homology to CDCrel-1, coimmunoprecipitates with Parkin from human substantia nigra lysates and is also ubiquitinated by Parkin *in vitro* (Choi et al., 2003). (Beites et al., 1999). Septins are evolutionary conserved proteins with essential functions in cytokinesis, and throughout the cell cycle. These small proteins likely with GTPase activity seem to function in membrane transport and exocytosis (Field and Kellogg, 1999).

D.2 Cyclin E

A number of RING proteins including Skp1, Cullin and F-box (SCF) complexes have been shown to function within multiprotein ubiquitin ligase complexes (Joazeiro and Weissman, 2000; Patton et al., 1998). Parkin is part of a SCF complex containing hSel-1 and Cullin-1 (Staropoli et al., 2003). This complex auto-ubiquitinates Parkin and also ubiquitinates Cyclin E via HSel-1 (Staropoli et al., 2003). Cyclin E is involved in kainite-induced excitotoxicity and primary neuronal apoptosis. Parkin knockdown by RNA interference results in the accumulation of Cyclin E in the primary cerebellar granule cells, while Parkin overexpression inhibits kainite-induced Cyclin E apoptosis (Staropoli et al., 2003).

D.3 α- and β-Tubulin

It was shown that Microtubule-associated protein 1A (MAP1A) binds Parkin in a temperature-sensitive manner with a higher coimmunoprecipitation at 37^{o}C than at 4^{o}C (Feng and Lin, 2001). This suggested that Parkin may be binding to MAP1A via microtubules, as polymerization of microtubules is known to be more efficient at

37^{o}C. Parkin was later identified as a tubulin-binding protein and an E3 ligase for α- and β-Tubulins (Ren et al., 2003). Interestingly, Parkin overexpression stabilized microtubules against colchicine-induced depolymerization (Yang et al., 2005).

***Drosophila parkin* null mutants**

Animal models of PD have facilitated the study of the mechanisms and processes involved in the pathogenesis of the disease. Till date, none of the animal models have been able to recapitulate all the hallmark pathologies and symptoms (Auluck and Bonini, 2002; Hamamichi et al., 2008). These models are generated via knockdown or mutagenesis of genes implicated in PD or by treatment with environmental toxins like 1-methyl, 4-phenyl-1,2,3,6-tetrahydropyridine and paraquat (Dauer and Przedborski, 2003). In our study, we use a *Drosophila* model of PD. To explore the biological role of *parkin*, a series of mutations have been generated in the *Drosophila* ortholog of *parkin*, including deletion, nonsense and missense mutations. Two lines of *parkin* null mutant flies were generated by insertional mutagenesis with activated EP element transposons by two independent groups (Greene et al., 2003; Pesah et al., 2004). In one line (used in our study), *parkin*-deficient males are sterile but the females are reported to be fertile (sterile in our hands) (Greene et al., 2003). Male sterility was further studied and was found to be derived from a spermatid individualization defect at a late stage of spermatogenesis, which may be a result of defective Nebenkern formation and/or function (Greene et al., 2003). The Nebenkern is a specialized mitochondrial derivative in the mature sperm. Ultrastructural analysis of *parkin* mutant males revealed severe disruption of the Nebenkern integrity. In contrast to this study, the other group reported both male and female sterility with homozygous *parkin* deletion (Pesah et al., 2004). They also reported an increased sensitization to oxidative stress and a 30% lower mass of *parkin* mutants in comparison to wild type controls. A reduced cell size and cell number accounted for this mass reduction.

Parkin-deficient flies show a reduced longevity and a partially penetrant downturned wing phenotype (Greene et al., 2003). Locomotor activity assays revealed severe defects in flight and climbing activity. These locomotor defects derive from apoptotic cell death of mitochondria and muscle subsets. Histological analysis of the major flight muscles from *parkin* mutants showed an extensive disruption of muscle

integrity, which was consistent with their inability to fly. Ultrastructural analysis of the flight muscles demonstrated a disintegration of the mitochondria with fragmented cristae which indicate that Parkin might regulate mitochondrial integrity. Similar to *parkin* knockout mice (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004; Perez and Palmiter, 2005; Von Coelln et al., 2004b), no significant dopamine neuron loss was observed in the mutant flies (Greene et al., 2005; Greene et al., 2003; Pesah et al., 2004), notwithstanding the observation of a 20% loss in one study involving only one subset of the dopamine neuron clusters in the fly brain (Whitworth et al., 2005). Transcriptional analysis indicated that genes involved in the innate immune response and oxidative stress components are upregulated in the *parkin* mutant animals (Greene 2005). A genetic screen for mutations in genes that either enhance or suppress the lethality of *Drosophila parkin* mutants revealed that loss-of-function mutations in oxidative stress components, such as glutathione S-transferase S1 enhance the Parkin mutant phenotype (Greene et al., 2005). The lifespan of the mutant flies is further reduced when they are grown on environmental toxins like rotenone or paraquat, which generates toxic free radicals *in vivo* (Pesah et al., 2004).

1.2.2 α-Synuclein (PARK 1)

α-Synuclein (also known as NACP, Synuclein-1 or Synelfin) was first discovered in the *Torpedo* (electric eel) electrical organ as a vesicle-associated component (Clayton and George, 1999) and was identified as the precursor protein of the non-amyloid β component of the senile plaques of Alzheimer's disease (Iwai, 2000). α-Synuclein has 140 amino acids and consists of three domains, including an amino-terminal amphipathic lipid binding α-helix, a hydrophobic β-amyloid binding domain that encodes the non-Aβ component (NAC) and an acidic carboxy-terminal tail.

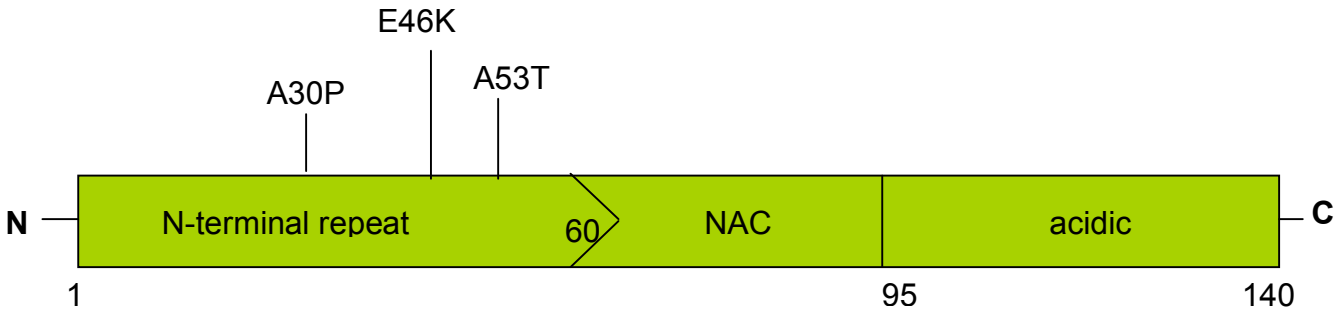

Figure 3: Schematic representation of α-Synuclein. The N-terminal region consists of 7 imperfectly conserved KTKEGV repeats. This motif has unknown physiological significance. The NAC domain is extremely hydrophobic and the C-terminal region is negatively charged. The three known missense mutations are localized to the N-terminal repeat region.

Either oxidative stress or mutations of *α-synuclein* may lead to the formation of highly toxic protein aggregates or fibrils, ultimately resulting in neuronal cell death. Missense mutations in the *α-synuclein* gene have been shown to be a cause of rare forms of autosomal dominant familial PD. One of the first identified genetic causes of PD is the missense mutation A53T in the *α-synuclein* gene in a large Italian and several smaller Greek kindreds. (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). Thereafter, two other missense mutations were identified; A30P and E46K (Kruger et al., 1998; Zarranz et al., 2004). Additionally, a triplication of the wild type *α-synuclein* gene resulting in α-Synuclein overexpression is also associated with familial PD (Singleton et al., 2003), indicating that both the composition and dosage of α-Synuclein appear to play roles in the development of PD. Oxidative stress has been shown to promote the aggregation of α-Synuclein in several studies (Hashimoto, M. 1999, Neuroreport 10: 717-721. Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro) (Ostrerova-Golts, N. 2000. J. Neurosci 20: 6048-54) (Paxinou, E. 2001, J.Neurosci 21: 8053-61) (Sherer, TB 2002, J. Neurosci 22: 7006-15). Modifications in the primary structure of the α-Synuclein protein may enhance its oligomerization and subsequent fibril formation (Conway et al., 1998).

Localization and putative functions of α-Synuclein

The location of α-Synuclein is predominantly in the brain and in presynaptic terminals but its normal physiological functions is largely unknown (Clayton and George, 1999; Jakes et al., 1994; Kahle et al., 2000b), though it appears to be involved in the biosynthesis of dopamine, synaptic vesicle recycling (Baptista et al., 2003; Perez et al., 2002) and synaptic plasticity (Clayton 1999). α-Synuclein associates with lipid membranes (Jo et al., 2000) and co-localizes with Synaptophysin and SNAP-25, which promote vesicle docking and fusion (Irizarry et al., 1996; Withers et al., 1997). *In vitro* studies revealed that α-Synuclein can bind these structures through its N-terminal repeat domain, thereby causing dramatic alterations in its secondary structure (Davidson et al., 1998; Jensen et al., 1998). This led to the hypothesis that α-Synuclein has an important function in vesicular transmitter release and synaptic plasticity (Clayton and George, 1998). Different animal models have been created to investigate how α-Synuclein causes PD. α-Synuclein knockout mice display neurodegeneration along with altered compartmentalization of presynaptic dopamine and decreased capacity of dopamine storage pool (Yavich et al., 2004). In addition the reduction in the quantal release of dopamine that usually occurs post repetitive stimulation of the same neurons was inhibited in mice lacking α-Synuclein while the dopamine-dependent response to amphetamine was increased (Abeliovich et al., 2000). These results are indicative of the role of α-Synuclein in the regulation of dopamine homeostasis. However, the involvement of α-Synuclein is not only restricted to dopamine neurons, as its loss has been shown to alter synaptic transmission at glutamatergic synapses as well (Martin et al., 2004). α-Synuclein transgenic flies display a selective dopaminergic neuronal loss and the presence of Lewy body-like inclusions in the neurons (Feany and Bender, 2000). Finally, several studies point towards α-Synuclein's role as a molecular chaperone owing to its 40% homology with the cytoplasmic chaperone 14-3-3 (Kim et al., 2000; Ostrerova et al., 1999).

α-Synuclein aggregation and toxicity

α-Synuclein has a high propensity to aggregate owing to its hydrophobic non-amyloid-β domain. α-Synuclein is transformed from its normal, soluble form to a disease-causing fibrillar state in many neurodegenerative disorders, which are

collectively referred to as α-synucleinopathies (Galvin et al., 2001; Goedert, 1999; Spillantini and Goedert, 2000). While the C-terminal of α-Synuclein is a key regulator of its aggregation *in vivo* (Li et al., 2005), the internal hydrophobic NAC region is capable of fibril formation *in vitro* (Han et al., 1995). The fibrils formed are similar to those observed in Lewy bodies, particularly in the A53T mutation (Conway et al., 1998). The potentially neurotoxic protofibrils formed may damage vesicular membranes by forming pore-like channels (Lashuel et al., 2002; Volles and Lansbury, 2002), similar to some bacterial toxins (Ding et al., 2002; Volles et al., 2001) and permeabilize the membranes of synaptic vesicles and other organelles, resulting in leakage of calcium and dopamine which are known to be cytotoxic at high levels (Caughey and Lansbury, 2003). This provided a mechanism of how protein fibrilization can cause cell death. Interestingly, the dopamine-α-Synuclein adducts stabilize the protofibrils (Conway et al., 2001). A pathological modification involving the phosphorylation of Ser129 in α-Synuclein was shown to promote aggregation besides forming a major component of Lewy bodies (Anderson et al., 2006; Smith et al., 2005). The G-protein-coupled receptor kinase 5 has been recently shown to be responsible for this phosphorylation (Arawaka et al., 2006). α-Synuclein has been reported to directly bind the human dopamine transporter in cotransfected cells (Lee et al., 2001) which may result in elevated dopamine levels and subsequent neuronal death as dopamine may participate in oxidative stress generating reactions, since both dopamine auto-oxidation and its metabolism produce reactive oxygen species (ROS). In addition, α-Synuclein inhibits of Tyrosine hydroxylase (Perez et al., 2002). Several studies link mutated α-Synuclein to an increased sensitivity of cells to proteasomal inhibitors by a decreased protesomal function (Petrucelli et al., 2002; Stefanis et al., 2001; Tanaka et al., 2001). Synphilin-1 binds to α-Synuclein *in vivo* and this association promotes the formation of intracellular inclusions (Engelender et al., 1999). In addition, it is suggested that Synphilin-1 regulates the synaptic functions of α-Synuclein, possibly by anchoring α-Synuclein to vesicular membranes (Ribeiro et al., 2002).

α-synuclein was the first gene shown to be associated with a heritable form of PD and was also the first of all PD-related genes to be studied in a *Drosophila* model of PD. Loss of dopamine neurons was observed in a transgenic *Drosophila* model of PD

caused by mutant α-Synuclein (Feany and Bender, 2000) in contrast to observations with several lines of *α-synuclein* transgenic mice which failed to develop dopamine neuron loss (Fernagut and Chesselet, 2004). On the other hand, rats overexpressing mutant synuclein, A30P (Klein et al., 2002; Lo Bianco et al., 2002) or A53T (Kirik et al., 2003), developed a progressive loss of dopaminergic neurons.

1.2.3 PINK1 (PARK6)

The ubiquitously expressed *PINK1* transcript encodes a 581 amino acids protein (Unoki and Nakamura, 2001) with a mitochondrial targeting motif at the N-terminal and a highly conserved protein kinase domain (156-509 amino acids) (Rohe et al., 2004; Valente et al., 2004), which is homologous to the serine/threonine kinases of the calcium/calmodulin family. Known mutations in *PTEN-induced kinase 1* (*PINK1*), associated with the PARK6 locus which result in early-onset autosomal-recessive PD include a W437Stop, nonsense and a G309D, missense mutation within the PINK1 kinase domain (Valente et al., 2004) and an insertion outside the kinase domain causing a frameshift and truncation at the C-terminus of PINK1 (Rohe et al., 2004). The mutants have an increased susceptibility to cellular stress. The wild type PINK 1 may prevent PD by protecting neurons from stress-induced mitochondrial dysfunction through phosphorylation of mitochondrial proteins (Valente et al., 2004). Loss-of-function mutations in the *Drosophila PINK1* ortholog results in morphological abnormalities of the mitochondria and apoptosis in testes ad flight muscles with dopaminergic neuronal loss in some cases (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Parkin overexpression rescues the PINK1 phenotype but the vice versa is not true. This indicates a functional relationship between the two genes implicated in PD, suggesting that *PINK1* functions upstream of *parkin* (Kitada et al., 2007).

1.2.4 Ubiquitin C-terminal hydroxylase L1 (PARK5)

Ubiquitin C-terminal hydroxylase L1 (UCH-L1) is a thiol protease that produces ubiquitin possibly by cleaving ubiquitin polymers or by releasing ubiquitin from cellular adducts with glutathione or amine (Larsen et al., 1998; Larsen et al., 1996). An autosomal dominant missense mutation (I93M) in the gene encoding UCH-L1 was first identified in two siblings having a strong family history of PD (Leroy et al.,

1998). UCH-L1 is an abundant neuronal enzyme that makes up 1-2% of the total brain protein, with an unknown function (Wilkinson et al., 1989). Quite interestingly, UCH-L1 has two enzymatic activities with opposite effects, both of which may play a role in proteasomal protein degradation: a beneficial hydrolase activity and a partly pathogenic dimerization-dependent ligase activity which causes the accumulation of α-synuclein in cultured cells. The I93M mutation decreases the *in vitro* hydrolytic activity of UCH-L1 (Liu et al., 2002). UCH-L1 is also present in Lewy bodies of sporadic PD (Lowe et al., 1990) and I93M mutant was shown to be able to produce inclusions (Ardley et al., 2004). A S18Y polymorphism was also discovered in the UCH-L1 protein and subsequently found to be linked to a decreased susceptibility to PD (Levecque et al., 2001; Maraganore et al., 1999; Satoh and Kuroda, 2001), possibly because it reduces the ligase activity of UCH-L1 while promoting its hydrolase activity (Liu et al., 2002).

1.2.5 DJ-1 (PARK7)

PARK7, which encodes a protein DJ-1 is yet another locus linked to early-onset recessive PD. Various mutations identified in affected individuals include exonic deletions, truncations and point mutations (L166P and M26I) (Abou-Sleiman et al., 2003; Bonifati et al., 2003; Hague et al., 2003; Hedrich et al., 2004). DJ-1 was identified as a protein that transforms mouse NIH3T3 cells in cooperation with activated ras (Nagakubo et al., 1997). Apart from playing a direct role in dopamine neuron physiology (Goldberg et al., 2005), DJ-1 is a component of a cAMP-responsive multiprotein complex that stabilizes (Hod et al., 1999), and it also functions as a positive regulator of the androgen receptor, antagonizing the function of negative regulators, such as DJ-1-binding protein. The human DJ-1 gene, which is comprised of eight exons, is mapped to 1p36.2–36.3, where many chromosome aberrations in cancer have been reported (Taira et al., 2001). DJ-1 was found to be more strongly expressed in the testis than other tissues and also in sperm.

DJ-1 plays the role of a redox-sensitive molecular chaperone *in vitro* and in cells, granting protection against ROS-generated toxicity by getting induced in an oxidative environment and inhibiting the aggregate formation of α-Synuclein (Schapira, 2008;

Shendelman et al., 2004). Also, overexpression of DJ-1 reduces, while DJ-1 knockdown by RNA interference enhances, the susceptibility of cells to oxidative stress (Taira et al., 2004). DJ-1 dimerization is essential for its function and the L166P mutation destabilizes DJ-1 by preventing dimer formation (Macedo et al., 2003; Moore et al., 2003), abolishing DJ-1 chaperone activity (Shendelman et al., 2004). Different studies in DJ-1-null mice have shown that DJ-1 protects against neuronal oxidative stress and that its loss may lead to PD by conferring hypersensitivity to dopaminergic insults (Goldberg et al., 2005; Kim et al., 2005). The *Drosophila* genome encodes two orthologs of the human *DJ-1* gene, *DJ-1a* and *DJ-1b*. while DJ-1b is expressed ubiquitously, DJ-1a is predominant in the testes (Menzies et al., 2005; Meulener et al., 2005; Park et al., 2005).

1.2.6 Leucine-rich repeat kinase 2 (PARK8)

Leucine-rich repeat kinase 2 also known as LRRK2 or Dardarin is a large protein and a member of the leucine-rich repeat kinase family which in humans is encoded by the *LRRK2 (PARK8)* gene. Dominant mutations of LRRK2 are the most common cause of inherited PD. Several missense mutations and one putative splice site mutation in the *PARK8* gene are linked to the development of PD (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). The pathological markers of the disease vary significantly (Brice, 2005; Wszolek et al., 2004; Zimprich et al., 2004). LRRK 2 is present largely in the cytoplasm but also associates with the mitochondrial outer membrane. It is expressed weakly throughout the brain, with slightly elevated levels in the putamen and the substantia nigra regions. The protein consists of 2527 amino acids and is encoded by 51 exons, encoding multiple conserved domains with an ankyrin repeat region, a leucine-rich repeat (LRR) domain, a kinase domain, a DFG-like motif, a RAS domain, a GTPase domain, an MLK-like domain, and a WD40 domain. (Paisan-Ruiz et al., 2004; Shen, 2004). LRRK2 is known to interact with the RING2 domain of Parkin and is hypothesized to be responsible for the phosphorylation of α-Synuclein and Tau (Shen, 2004).

1.3 Mechanisms of cell death in PD

Although about 95% cases of PD seem to be sporadic, inherited cases have resulted in discovery of PD-linked genes. The genes encoding α-Synuclein, Parkin, DJ-1,

PINK1, UCH-L1 and LRRK2 are known to carry mutations that cause PD. Several homozygous and compound heterozygote mutations may result in loss of protein function, altered protein stability and/or localization or aggregation of accumulated proteins. Key mechanisms contributing to the pathogenesis of PD include mitochondrial impairment, oxidative stress, protein mishandling and altered dopamine homeostasis.

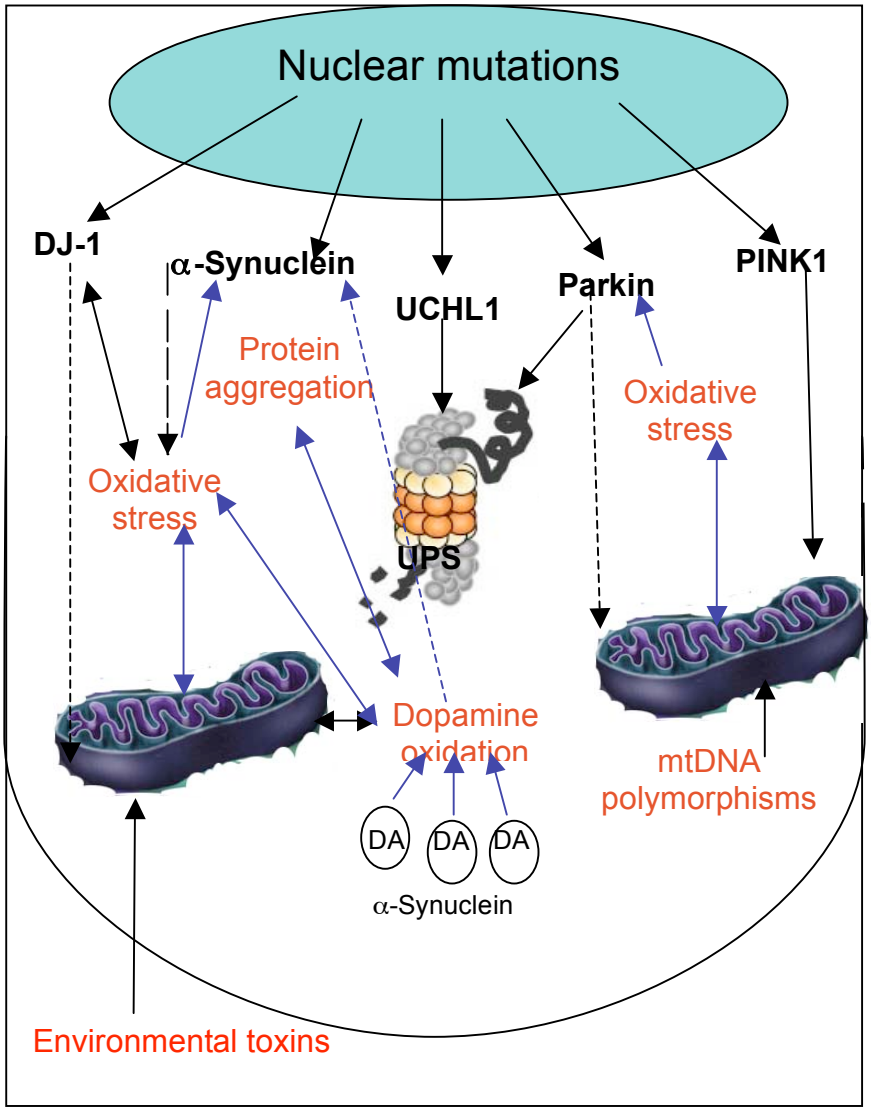

Figure 4: Genetics and molecular mechanisms of PD. Black arrows indicate putative primary causes of PD; dashed arrows indicate effects that are secondary. Blue arrows indicate mechanisms of PD that are secondary to primary causes. mtDNA, mitochondrial DNA. According to Greenamyre and Hastings, 2004.

1.3.1 Oxidative stress and mitochondrial dysfunction

Post mortem studies have repeatedly implicated oxidative stress in PD pathogenesis (Valente et al., 2004). Gene knockout of parkin mouse and flies show increased oxidative stress and mitochondrial dysfunction (Palacino et al., 2004; Pesah et al., 2004). Reactive oxygen species (ROS) can lead to functional alterations in biomolecules like proteins, lipids and DNA. Immunocytochemical analyses revealed an increased level of oxidized RNA and DNA in nigral neurons of PD patients (Alam et al., 1997; Zhang et al., 1999). Leading candidates for generation of oxidative stress include dopamine metabolism, mitochondrial dysfunction, increased free iron levels, impaired free radical defense and environmental chemicals (Foley and Riederer, 2000). ATP production in the mitochondria is regulated by respiratory chain complexes that are involved in the transfer of electrons to oxygen, resulting in the generation of free ROS. While cells tolerate normal levels of ROS, its overproduction combined with insufficient antioxidant defense mechanisms can induce mitochondrial dysfunction, which in turn may enhance the production of more deleterious ROS, further worsening the mitochondrial disturbed state, thus creating a vicious cycle (Tritschler et al., 1994). Nigrostriatal dopaminergic neurons might be particularly exposed to oxidative stress even under physiological conditions, probably due to redox cycling of catechols, resulting in the generation of detrimental ROS. Dopamine (DA) readily oxidizes and reacts with proteins, nucleic acids and lipids forming neurotoxic derivatives, including 6-hydroxydopamine, DA-quinones, superoxide radicals and hydrogen peroxide (Lotharius and Brundin, 2002; Olanow and Tatton, 1999) and its interaction with intracellular iron results in formation of toxic oxygen radicals (Sulzer and Zecca, 2000). Additionally, DA turnover pathway involving the enzymatic deamination of dopamine by monoamine oxidase generates hydrogen peroxide along with the non-toxic dopamine metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid (Graham, 1978). To escape from cytosolic DA stress, DA neurons have evolved several defense mechanisms which include feedback inhibition of tyrosine hydroxylase, metabolism by monoamine oxidase, sequestration of DA in synaptic vesicles as well as reduction of DA quinone by glutathione (Nakamura et al., 2001). The quantification of the reaction products of ROS in post-mortem PD brain tissue serves as an indirect index of oxidative stress induced cell damage (Foley and Riederer, 2000).

1.3.2 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) caters to multiple cellular functions. Accumulation of misfolded proteins within the ER disturbs its normal functions, induces a highly specific and evolutionarily conserved unfolded protein response (UPR) and is the major cause of ER stress (Kaufman, 1999). ER stress is aimed initially at compensating for damage but can eventually trigger cell death if ER dysfunction is severe or prolonged. The UPR upregulates ER chaperone and folding catalysts and prevents further protein aggregation by attenuating protein synthesis leading to the activation of the ER-associated protein degradation (ERAD) for eliminating the harmful aggregates (Szegezdi et al., 2003). The mechanisms by which ER stress leads to cell death remain enigmatic, however, several mechanisms including direct activation of proteases, kinases, transcription factors, and Bcl-2–family proteins and their modulators have been implicated. ER stress has been demonstrated to produce mitochondrial dysfunction by interfering with the expression and assembly of the cytochrome c oxidase (Hori et al., 2002). Also, the steady state levels of nuclear-encoded cytochrome c oxidase were reduced and the mitochondria-encoded subunit was rapidly degraded (Hori et al., 2002). UPR causes the ER-resident kinases to induce genes encoding ER chaperones and nuclear transcription factors that ultimately result in either ER stress reduction, achieved by phosphorylation of the eukaryotic initiation factor-2α (eIF2α) resulting in the attenuation of protein synthesis (Harding et al., 1999) or cell death if the damage is beyond repair (Mori, 2000).

In a cell culture model of PD, ER stress was inferred from the identification of phosphorylated protein kinase R-like ER kinase (PERK) and Inositol-requiring-1 (IRE1) in rat neuronal PC12 cells upon 6-hydroxydopamine exposure, which was taken up by the dopamine transporters and resulted in selective dopamine neuron death (Ryu et al., 2002). PERK (PKR-like ER kinase) is a serine-threonine protein kinase, the catalytic domain of which shares substantial homology to other kinases of the eukaryotic initiation factor 2 (eIF2) family (Harding et al., 1999; Shi et al., 1998). UPR was evoked by activated PERK and IRE1 (Ryu et al., 2002). Familial mutations in the ER-associated E3 ubiquitin ligase Parkin have also been associated with ER stress–induced cell death and are found in patients with familial PD (Dawson and Dawson, 2003; Takahashi et al., 2003). Importantly, Parkin expression is induced by ER stress, suggesting a role for it in adaptation to ER stress. Knockdown of *parkin* in

cell culture resulted in accumulation of its substrate Pael-R, which caused ER stress and cell death (Imai et al., 2001). These studies indicate that ER stress may be another mechanism underlying PD pathogenesis.

II) Metal-responsive transcription factor-1 (MTF-1)

MTF-1 (also metal-regulatory transcription factor-1), a zinc-finger transcription factor, is evolutionarily conserved from insects to mammals and activates cellular defense mechanisms in response to metals like zinc, copper and cadmium (Lichtlen and Schaffner, 2001; Westin and Schaffner, 1988). Its DNA binding domain consists of six C2H2 zinc fingers (Dalton et al., 1997; Radtke et al., 1993) and thus zinc is essential for the binding of MTF-1 to DNA. The DNA-binding motif is termed metal response element (MRE) and has a core consensus sequence of TGCRCNC (Stuart et al., 1984; Stuart et al., 1985). Copper and cadmium regulate MTF-1 indirectly by displacing zinc from zinc-loaded metallothioneins (MTs) or other zinc-binding proteins *in vitro* (Bittel et al., 1998; Zhang et al., 2003). However, *in vivo*, MTF-1 is subject to more complex regulation that includes phosphorylation and nuclear translocation (Saydam et al., 2002; Saydam et al., 2001). In quiescent cells, MTF-1 is predominantly cytoplasmic, but is translocated to the nucleus upon metal load or other stress conditions. In the nucleus, MTF-1 binds to the MREs and activates its target genes including metallothioneins, zinc transporters and placenta growth factor (Cramer et al., 2005; Langmade et al., 2000).

The MTF-1 regulatory protein has been characterized in human (Brugnera et al., 1994), mouse (Radtke et al., 1993), pufferfish (Auf der Maur et al., 1999), *Drosophila* (Zhang et al., 2001), zebrafish (Chen et al., 2002), carp (Ferencz and Hermesz, 2008) and capybara (Lindert et al., 2008), but has not been found in *C. elegans* or yeast. Human and mouse MTF-1 show 93% sequence identity, the truncated C-terminus of the mouse notwithstanding. Vertebrate and insect MTF-1 show only 39% sequence similarity of the full-length protein, most of which is accounted for by a 78% similarity in the zinc-finger region.

Human MTF-1 harbors a non-canonical nuclear localization signal (NLS) sequence overlapping with the zinc-finger domain (Lindert et al., 2009) and a nuclear export

signal (NES) sequence that overlaps with the major activation domain (Saydam et al., 2001). MTF-1 also contains three different transactivation domains C-terminal to the DNA binding domain: an acidic, a proline-rich, and a serine/threonine –rich domain.

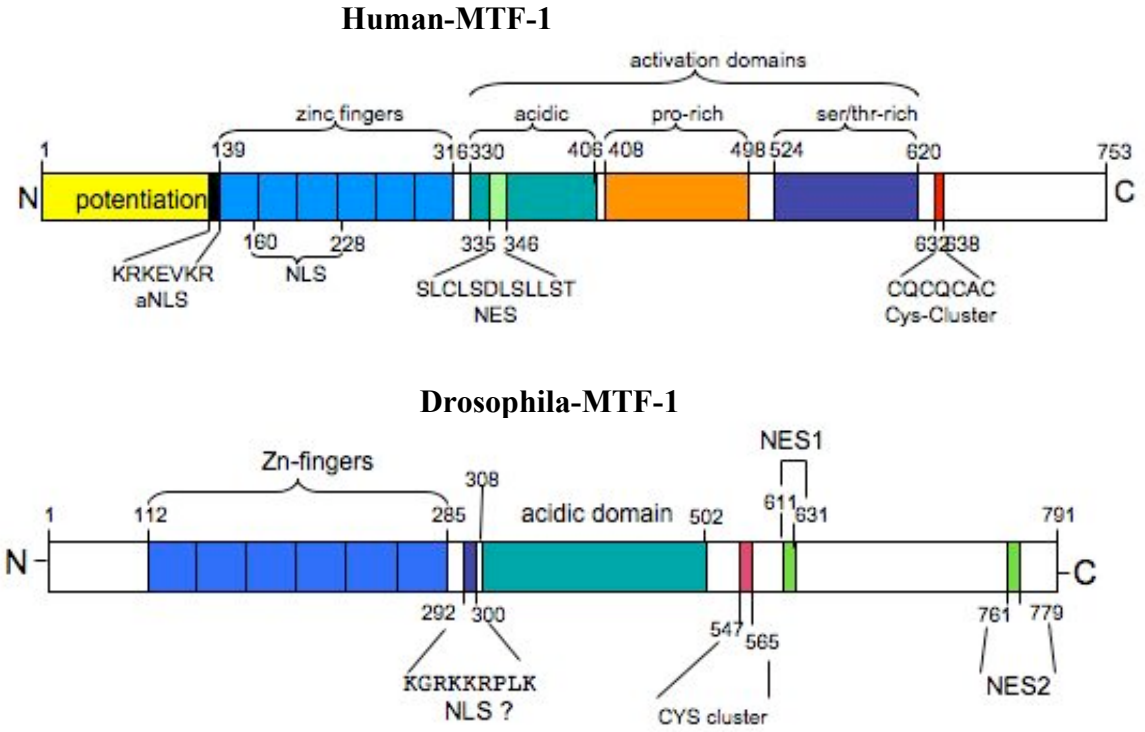

Figure 5: Schematic view of the functional domains of human-MTF-1 (top) and Drosophila-MTF-1 (below). Domains of human-MTF.1 include a potentiation domain (yellow), C_2H_2-zinc finger domain (blue), acidic activation domain (green), proline-rich domain (orange) and serine/threonine-rich domain (dark blue), auxiliary nuclear localization signal (aNLS) (black box on left), nuclear export signal (NES) (light green box), cysteine-cluster (red box). Drosophila-MTF-1 domains indicate the zinc-finger domain (blue), putative NLS (dark blue), acidic-domain (green), NES1 and 2 (light green), cys-cluster (red).

Targeted disruption of the MTF-1 gene has been achieved in the mouse and in *Drosophila melanogaster*. MTF-1 knockout mice die at the embryonic stage (E13-E14) due to liver degeneration (Gunes et al., 1998). *Drosophila* MTF-1 (dMTF-1) null mutants are viable but highly sensitive to heavy metal stress and also to copper starvation (Egli et al., 2003). dMTF-1 has the unique ability to handle both extremes, namely, copper overload and copper scarcity (Selvaraj et al., 2005). Upon copper stress, it activates the metallothionein genes, while under copper depletion condition it upregulates the copper importer Ctr1B.

Metallothioneins and other MTF-1 target genes

The best characterized target genes of MTF-1 are the metallothioneins. Metallothioneins are small proteins of 61-68 amino acids with a very high cysteine content (up to 30%) (West et al., 2008). Four metallothionein genes have been identified in the mouse. Mt-1 and Mt-2 are ubiquitously expressed and are highly inducible in response to various stress conditions (heavy metal ions, hormones, cytokines, ROS, inflammation). Mt-3 expression is restricted to the central nervous system and Mt-4 to the squamous epithelia, whereby the Mt3 gene contains MRE like sequence motifs but appears not to be regulated by MTF-1. Mice with a combined knockout of Mt-1 and Mt-2 are viable, in contrast to the MTF-1 knockout mice, however, they are sensitive to cadmium and zinc (Kelly et al., 1996; Masters et al., 1994; Michalska and Choo, 1993). In humans, MT-1 has diverged into a family of 7 genes (Mt-1A, -B, -E, -F, -G, -H and –X) while the other isoforms are represented by only one gene each, Mt-2A, Mt-3 and Mt-4 (Sadhu and Gedamu, 1988; Stennard et al., 1994; West et al., 1990). There is increasing evidence for a protective role of metallothioneins against neurodegeneration and CNS injury (West et al., 2008).

In *Drosophila melanogaster* there are at least four metallothioneins, designated MtnA-D, which are preferentially expressed in regions of high metal content, such as the larval intestinal tract and the midgut. They are induced by cadmium, zinc, copper, silver and mercury in an MTF-1 dependent manner (Egli et al., 2006). The quadruple knockout of the four Mtns leads to a severely reduced lifespan in males. Furthermore, flies are highly sensitive to copper, cadmium and to a lesser extent to zinc during development (Egli et al., 2006). Recently a putative fifth Mtn, MtnE, located proximal to MtnD was identified (Susan Celniker, Lawrence Berkeley National Laboratory) and is currently being investigated in our Lab (L. Atanesyan, H. Hua, O. Georgiev and WS, unpublished).

In fungi, metallothionein genes (CUP1 and CRS5 in *Saccharomyces cerevisiae*) are regulated by copper-sensitive transcription factors like Ace1 which have no sequence homology to MTF-1 (Rutherford and Bird, 2004). The metallothionein regulating transcription factor of *Candida glabrata* is Amt1 and that of *Yarrowia lipolytica* (dimorphic yeast) is Crf1. All these factors display a significant sequence similarity in their N-termini (Garcia et al., 2002).

Microarray studies done for the identification of novel target genes of MTF-1 in MTF-1 knockout mice, revealed α-fetoprotein, the liver-enriched transcription factor C/EBPα and tear lipocalin as new target genes, all of them being involved in stress response (Lichtlen and Schaffner, 2001). A zinc transporter, ZnT1 is also under MTF-1 control in the mouse (Langmade et al., 2000). As part of a screen carried out for zinc-responsive transcripts, Kruppel-like factor 4, hepatitis A virus cellular receptor 1 and complement factor B were also proposed as MTF-1 target genes (Kindermann et al., 2005). MTF-1 was shown to be essential for the basal expression of selenoprotein W (Sepw1), a putative antioxidant protein having a glutathione binding site (Wimmer et al., 2005). With regard to stress response, MTF-1 regulates cadmium-induced expression of N-myc downstream regulated gene 1 (Ndrg1) as well as the cadmium response of the cysteine- and glycine-rich protein 1 gene (Csrp1) that is involved in cytoskeletal organization (Wimmer et al., 2005). MTF-1 downregulates metal importers, namely, the zinc transporter, Slc39a10 (ZIP10) (Kaler and Prasad, 2007) and in *D. melanogaster* the copper transporter Ctr1B. Additionally, transcription of the gene encoding hypoxia inducible placental growth factor, a pro-angiogenic protein, was shown to depend on MTF-1 (Green et al., 2001). Furthermore, siRNA knockdown experiments in cell culture suggested a cooperation of MTF-1 with Sp1 in the copper dependent positive regulation of the prion protein PrP gene (Bellingham et al., 2009). Misfolding of PrP causes transmissible spongiform encephalitis, a neurodegenerative disorder termed Creutzfeldt-Jakob disease in humans, BSE in cattle and scrapie in sheep. Growing evidence points towards a role of PrP in copper homeostasis (Brown et al., 1997; Pauly and Harris, 1998; Ruiz et al., 2000; Wadsworth et al., 1999). A search for MTF-1 target genes in *Drosophila* revealed results similar to mammals. MTF-1 targets include metallothioneins (MtnA-D), the zinc efflux transporter ZnT35C, the ATP binding cassette (ABC) transporter CG10505 and ferritins, which play a major role in iron homeostasis (Egli et al., 2003; Yepiskoposyan et al., 2006). Furthermore, dMTF-1 is implicated in the copper-responsive expression of DmAtp7, the Drosophila ortholog of the Menkes and Wilson's disease proteins Atp7A and Atp7B, in the larval midgut (Burke et al., 2008).

All these verified and inferred target genes highlight the central role of MTF-1 in metal homeostasis, defense against various stresses like heavy metal toxicity, heat shock, ROS and hypoxia as well as in angiogenesis and neuroprotection.

III) Zinc homeostasis

Zinc is an essential heavy metal that functions as a structural component in a number of proteins, including transcription factors and enzymes. Accordingly, zinc is important for several biological processes, such as nucleic acid metabolism, cell growth and proliferation, immune response and brain development. The concentrations of cellular zinc are tightly regulated. Uptake and efflux of zinc requires transporters which are grouped into two families based on their structure and functions. The ZIP protein family (also called as ZRT/IRT-related proteins) import zinc from extracellular space or intracellular vesicles into the cytoplasm (Eide, 2004). The ZnT proteins are involved in intracellular trafficking and excretion of zinc (Liuzzi and Cousins, 2004; Palmiter and Huang, 2004). In mammals, ZIPs are encoded by a solute carrier family 39A (SLC39A) genes and ZnTs (ZnT1-7) are encoded by solute carrier family 30A (SLC30A) genes (Palmiter and Findley, 1995). There are twelve ZIP genes identified so far in humans and three in mouse (Gaither and Eide, 2001). Under zinc-limiting conditions mice lacking ZIP1, ZIP2 or ZIP3 are embryonic lethal (Dufner-Beattie et al., 2006; Peters et al., 2007). Certain mutations in ZIP4 in humans cause a zinc deficiency disorder termed acrodermatitis enteropathica (AE) (Wang et al., 2002). Homozygous ZIP4 mutant mice die at early embryonic stages while heterozygous mutants are highly sensitive to zinc deficiency, similar to AE patients. Furthermore, female mice lacking functional ZnT4, which is expressed in breast epithelial cells, have low zinc levels in the milk which is fed to their pups, resulting in the death of the pups (Huang and Gitschier, 1997). In *Drosophila*, two ZIP genes have been identified, the *fear of intimacy foi* which is the counterpart of mammalian ZIP6/ZIP10 and *catsup* which is the counterpart of mammalian ZIP 7 (Mathews et al., 2005; Stathakis et al., 1999). One of the ZnTs, *ZnT35C* has been described in Drosophila (Yepiskoposyan et al., 2006). While *Drosophila* MTs preferentially bind copper and cadmium, mammalian MTs predominantly bind zinc, at least in non-metal stressed cells (Palmiter and Huang, 2004).

Zinc homeostasis in yeast is controlled primarily through the regulation of zinc uptake (Eide, 1997). *S. cerevisiae* has two separate uptake systems to obtain zinc from its environment, a high affinity transporter protein (Zrt1) and a lower affinity transporter (Zrt2), both of which are regulated by zinc availability; zinc limitation induces, and zinc-replete conditions repress their expression (Zhao et al., 1998). This zinc-responsive transcriptional regulation requires the regulatory protein Zap1, the function of which is repressed in high-zinc conditions (Zhao et al., 1998). Zap 1 binds to a conserved sequence, called the zinc response element (ZRE) of consensus 5'-ACCYYNAAGGT-3' which is present in the Zrt1, Zrt2, Zrt3 and ZAP1 promoters (Lyons et al., 2000).

References for Introduction

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Extended lifespan of *Drosophila parkin* mutants through sequestration of redox-active metals and enhancement of anti-oxidative pathways.

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Abstract

The mechanisms underlying neuron death in Parkinson's disease are unknown, but both genetic defects and environmental factors are implicated in its pathogenesis. Mutations in the *parkin* gene lead to autosomal recessive juvenile Parkinsonism (AR-JP). Here we report that compared to control flies, *Drosophila* lacking *parkin* show significantly reduced lifespan but no difference in dopamine neuron numbers when raised on food supplemented with environmental pesticides or mitochondrial toxins. Moreover, chelation of redox-active metals, anti-oxidants and overexpression of superoxide dismutase 1 all significantly reversed the reduced longevity of *parkin*-deficient flies. Finally, *parkin* deficiency exacerbated the rough eye phenotype of *Drosophila* caused by overexpression of the copper importer B (Ctr1B). Taken together, our results demonstrate an important function of *parkin* in the protection against redox-active metals and pesticides implicated in the etiology of Parkinson's disease. They also corroborate that oxidative stress, perhaps as a consequence of mitochondrial dysfunction, is a major determinant of morbidity in *parkin* mutant flies.

Introduction

A combination of mitochondrial dysfunction, oxidative stress and abnormal protein aggregation underlies the pathogenesis of PD ([Bueler, 2009], [Henchcliffe and Beal, 2008], [Cookson, 2005], [Dawson and Dawson, 2003], [Moore et al., 2005] and [Shen and Cookson, 2004]). About 10% of PD cases are heritable and caused by mutations in at least five genes ([Gasser, 2009], [Hardy et al., 2009] and [Hatano et al., 2009]). Parkin (PARK2) mutations lead to autosomal recessive juvenile Parkinsonism (AR-JP) ([Abbas et al., 1999] and [Kitada et al., 1998]). Parkin functions as an E3 ubiquitin ligase in the proteasome-mediated turnover of several proteins in vitro ([Corti et al., 2003], [Imai et al., 2001], [Shimura et al., 2000] and [Zhang et al., 2000]), some of which accumulate in the brains of AR-JP patients and/or mice carrying a targeted deletion of the Parkin gene ([Fukae et al., 2009], [Murakami et al., 2004] and [Periquet et al., 2005]). Ablation of Parkin in mice resulted in mitochondrial respiratory defects, decreased levels of anti-oxidant proteins (Palacino et al., 2004) and abnormalities in dopamine neurotransmission, but not loss of dopamine neurons ([Goldberg et al., 2003] and [Itier et al., 2003]). Recently, Parkin has been linked to DNA repair ([Kao, 2009a], [Kao, 2009b] and [Rothfuss et al., 2009]). Parkin was shown to enhance the replication, transcription and repair of mitochondrial DNA, thereby protecting mitochondrial DNA against oxidative damage (Rothfuss et al., 2009). *Drosophila* lacking Parkin display increased sensitivity to oxidative stress and severe structural mitochondrial abnormalities in muscle and germline tissues associated with apoptotic muscle degeneration and male sterility ([Greene et al., 2005], [Greene et al., 2003] and [Pesah et al., 2004]). There is controversy as to whether or not frank dopamine neuron death occurs in flies with *parkin* loss-of-function mutations. Limited dopamine neuron death restricted to the PPL1/DL1 cluster has been detected in 20-day-old *parkin* mutant flies (park25 allele) by tyrosine hydroxylase (TH) immunohistochemistry combined with whole-mount confocal microscopy (Whitworth et al., 2005). However, the original characterization of the same flies showed that dopamine neuron numbers determined in paraffin-embedded sections were normal, even in older *Drosophila* (30 days) (Greene et al.,

2003). Another line of *parkin* mutant flies (Pesah et al., 2004) also lacked dopamine neuron death, but the PPL1 cluster showing neuron loss (Whitworth et al., 2005) was not analyzed in this study. Although (Greene et al., 2003) and (Whitworth et al., 2005) used different methods to quantify TH neurons, it cannot be concluded that confocal microscopy is more likely to detect TH neuron loss than using paraffin-embedded sections. In fact, in α-synuclein transgenic flies, TH neuron loss was detected using paraffin-embedded sections (Feany and Bender, 2000), but not with whole-mount confocal microscopy (Pesah et al., 2005), although both groups used the same flies again. Finally, several groups failed to detect dopamine neuron loss in Parkin-deficient mice ([Goldberg et al., 2003], [Itier et al., 2003] and [Palacino et al., 2004]). Taken together, the data suggest that Parkin ablation may lead to neuron death in one of several dopamine neuron clusters in the *Drosophila* brain, while Parkin appears to be dispensible for dopamine neuron survival in mice.

Several epidemiological studies showed that long-term exposure to pesticides, such as rotenone and paraquat, correlates with an increased risk of developing idiopathic PD ([Abbott et al., 2003], [Baldi et al., 2003], [Gorell et al., 1998] and [Petrovitch et al., 2002]). Intravenous administration of an illicit drug, which contained 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), caused an acute and permanent state of Parkinsonism in humans (Langston et al., 1983). Moreover, excessive accumulation of iron in the substantia nigra has been implicated in PD pathogenesis ([Dexter et al., 1992], [Friedman et al., 2007] and [Zecca et al., 2004]). Free iron is toxic to cells as it acts as a catalyst in the formation of free radicals from hydrogen peroxide (H2O2) via the Fenton reaction (Orino et al., 2001). Because H2O2 is a normal product of the dopamine metabolism, dopaminergic neurons may be particularly vulnerable to accumulation of free iron. Support for increased free iron levels in PD comes form studies that showed reduced amounts of l-ferritin, which is important for iron sequestration (Harrison and Arosio, 1996), in PD compared to control brains ([Connor et al., 1995] and [Koziorowski et al., 2007]). In addition to iron, copper toxicity may play a role in various neurological disorders (Desai and Kaler, 2008). In Wilson's disease, increased copper

accumulation leads to liver dysfunction and neurological symptoms (Ala et al., 2007). Interestingly, patients with Wilson's disease often present with parkinsonism (Oder et al., 1993 W. Oder et al., Wilson's disease: evidence of subgroups derived from clinical findings and brain lesions, Neurology 43 (1993), pp. 120–124. View Record in Scopus | Cited By in Scopus (59)Oder et al., 1993) and deficits in dopamine neurotransmission have been reported (Barthel et al., 2003). Furthermore, neurological symptoms were shown to correlate with accumulation of copper in brain areas affected in PD, such as the basal ganglia and substantia nigra (Sudmeyer et al., 2006). This suggests that the nigrostriatal system may be particularly sensitive to copper accumulation. In agreement with this notion, long-term occupational exposure to copper was associated with an increased risk for PD (Gorell et al., 1997), and treatment of cultured dopaminergic neurons with copper led to mitochondrial defects, decreased dopamine content and cell death (Shi et al., 2008).

Because no single, unified mechanism leads to PD, it has been postulated that exogenous and/or endogenous stress factors may synergize with genetic defects to cause the precipitation of PD. This “gene–environmental factor interaction hypothesis” states that mutations in familial PD genes may increase the vulnerability to PD-associated environmental factors and toxins. Here, we hypothesized that *parkin* represents a genetic susceptibility factor in the vulnerability of flies to redox-active metals, pesticides and mitochondrial toxins implicated in PD. To test this hypothesis, flies carrying a null mutation (*park25/25*) of the *Drosophila* homologue of *parkin* (Greene et al., 2003) were raised on food supplemented with redox-active metals, metal chelators, rotenone, paraquat and MPP^+, and their survival was compared to *park25/+* control flies. The interaction of copper with *parkin* deficiency was further studied in flies overexpressing the copper importer Ctr1B. Finally we increased the anti-oxidant defense in flies by various means to corroborate the causative role of oxidative stress in the reduced longevity observed in *parkin*-deficient flies. Our results demonstrate that loss of Parkin can greatly increase the flies' vulnerability to environmental factors, mitochondrial toxins and certain redox-active metals implicated in the etiology of PD, and reinforce that

oxidative stress is a major pathway in causing the morbidity of *parkin*-deficient flies.

Materials and methods

Fly stocks and maintenance

All flies were maintained at 25 °C on a 12:12 h light–dark cycle. Unless specified otherwise, heterozygous *parkin* mutant flies (*w;+;park25/TM6B, w+*, hereafter referred to as *park25/+*) were used as controls for the homozygous *parkin* mutant flies (*w;+;park25/park25*, hereafter referred to as *park25/25*). *park25/25* is null mutation of the *Drosophila parkin* gene (Greene et al., 2003). The TM6B balancer in the control flies was confirmed to have no effect on any of the experiments performed (by having it crossed out). The *w;UAS-SOD1/Cyo;+* line expressing SOD1 under the mesoderm-specific twist-Gal4 driver (Greig and Akam, 1993) was received from the Bloomington stock center. These flies were crossed to the *w;+;park25/TM6B,w+* and *twi-Gal4;+;+* flies to obtain *twi-Gal4;UAS-SOD1;park25/+* animals, which were bred to obtain *twi-Gal4;UAS-SOD1;park25/25* flies. The Ctr1B coding region was amplified by PCR (with primers 5'-TGGACTAGATCTCAACATGGATCACGGCTCGGAT-3' and 5'-GTCAGT-AGATCTCTAAGGACAGCACTCGCT-3') from a Ctr1B cDNA clone and inserted into the SmaI site of pBluescript (Stratagene). The resulting construct was digested with EcoRI and NotI and the fragment was cloned between the EcoRI and NotI sites of the P element vector pUAST. The GMR-Ctr1B transgenic flies were generated with a Ctr1B ORF driven by the eye-specific GMR promoter/enhancer. The *w;GMR-Ctr1B/Cyo; park25/+* transgenic flies were obtained by crossing the *w;GMR-Ctr1B/Cyo* flies with *w;+;park 25/TM6B, w+* flies.

Preparation of fly food for oxidative stress, metal toxicity and metal chelator experiments

One liter standard fly food (normal food, NF) contained 55 g cornmeal, 10 g wheat flour, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent

nipagin (15% in ethanol). Normal food was supplemented with 200 μM rotenone, 10 mM paraquat and 25 mM 1-methyl-4-phenylpyridinium iodide (MPP+) (final concentrations, all from Sigma). For the metal toxicity/chelation and antioxidant experiments, normal food was supplemented with 500 μM ammonium ferric citrate, 500 μM copper (II) sulfate, 0.3 mM bathocuproine disulfonate (copper chelator), 0.1 mM bathophenanthroline sulfonated sodium salt (iron chelator), 20 or 50 μM silver nitrate, 15 mM N-acetyl-cysteine and 100, 250 or 500 mM ascorbic acid.

Lifespan determination

Lifespan (survival) experiments were carried out in triplicate. In each experiment, twenty 1- to 2-day-old flies per genotype were analyzed. Surviving flies were counted daily and transferred to fresh food vials every second day, with the exception of the SOD1 overexpression experiment where flies were counted only on the four indicated days. As a control, flies of the same genotype were grown on food lacking the toxins, metals, metal-chelating agents or antioxidants. The variations in the average lifespan of control flies in different experiments can be attributed to subtle experimental variations.

Exposure to hypoxia and heat shock

park25/+ and *park25/25* flies (six sets of 20 flies each) were exposed for 7 min to 0.5% oxygen at 25 °C in a closed hypoxia chamber, and the time to regain consciousness (ability to move) was recorded. To achieve a stable atmosphere of 0.5% oxygen, ambient air was mixed with nitrogen gas (Digamix M302 a-F, H Wösthoff e.H.G) and pumped into the chamber (VENT2 pump, EMKA Technologies) for 4 min at a flow rate of 25 l per minute. In the heat shock experiment, flies were incubated at 38 °C for 30 or 60 min and surviving animals were counted 24 h later.

Quantification of food uptake (gustatory assay)

For the adult gustatory assay, we performed a protocol similar to the one described by Hilliker and colleagues (Bahadorani et al., 2008). Briefly, newly eclosed flies were reared on normal food for 2–3 days before being starved for 18 h on Whatman paper soaked with distilled water. Subsequently, starved flies (n = 20) were transferred into a vial with fresh normal food (control) or chelator-supplemented food, both containing 0.2 % sulforhodamine B sodium salt (acid-red), and allowed to feed for 2 h. After 2 h of feeding, flies were decapitated and their bodies (without heads) were homogenized individually in 100 µl ice-cold buffer (0.32 M sucrose, 20 mM HEPES, 1 mM MgCl2, 0.5 mM PMSF, pH 7.4). 50 µl of the homogenate supernatants were transferred to separate wells of a microtiter (ELISA) plate and absorbance was measured at 565 nm (absorbance maximum for acid-red) using a multi-label counter (Wallac 1429, Perkin Elmer).

Quantification of brain ROS levels

Twenty heads dissected from adult flies were homogenized in 3 volumes of ice-cold buffer (0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, 0.5 mM PMSF, pH 7.4) and centrifuged for 20 min at 4 °C at 20,000g. Total protein concentration in the supernatant was measured with the Bradford method (Biorad kit) and adjusted to 0.4 µg/µl. 20 µl sample (8 µg protein) was mixed with 2 µl 40 µM DCF-DA in a fluorimeter cuvette (Hellma, 1.5 × 1.5 mm). Excitation and emission maxima of DCF are at 485 nm and 520 nm, respectively. The shutter size of the fluorimeter was fixed at 2.5 nm and sample fluorescence intensity was monitored for 45 min at 26 °C between 500 and 600 nm. Fluorescence intensity was recorded every 5 min. The maximum peak values were then read as the final value, by integrating the area under the peak of the graphs.

Analysis of eye phenotype

Flies were frozen at −20 °C and pictures of their eyes were taken with a scanning electron microscope (model JEOL JSM-630LV) in the imaging

facility of the University of Zurich. Ten eyes per genotype and treatment condition were analyzed.

Detection of TH-positive neurons in brain sections by DAB immunohistochemistry

Flies were lined up on a Heisenberg collar and fixed in Carnoy's solution (60% dried ethanol, 30% chloroform, 10% acetic acid) overnight at 4 °C (Heisenberg and Böhl, 1979). They were then dehydrated for 40 min in absolute ethanol, twice for 40 min in dried ethanol and twice for 60 min in n-butanol. Paraffin-embedded flies were decapitated, cut into 5-micron-thick sections and transferred to Super Frost Plus slides. Slides were placed in xylol for 2–5 min for de-waxing and then transferred to absolute ethanol for 2–5 min, before incubation in 95% ethanol for 2–3 min. Then they were placed into 70% ethanol for 2–3 min and the ethanol was washed out. Sections were fixed in 0.5% formaldehyde/phosphate-buffered saline (PBS) for 30 min, rinsed three times in PBS and blocked by incubation for 30 min in 1% bovine serum albumin/0.05% Triton X-100. After washing four times for 5 min in PBS/0.05% Triton X-100 they were incubated in PBS/1% bovine serum albumin (4 °C overnight) with 1:200 diluted monoclonal anti-tyrosine hydroxylase antibody specific for dopamine neurons (Chemicon, MAB 318). After five washes with PBS/0.05% Triton X-100, 1:1000 diluted secondary biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was added and the sections were incubated for 2 h at room temperature. Finally, they were washed five times with PBS and TH neurons were stained with 3,3'-diaminobenzidine (DAB) using the Vectastain Elite ABC Kit (Vector Laboratories).

Quantification of TH neurons in whole mount *Drosophila* brains by confocal microscopy

Fly brains were isolated and fixed for 2 h on ice in 100 mM Pipes/2 mM magnesium sulphate/1 mM EDTA/4% formaldehyde. They were rinsed twice with PBST (PBS/0.1% Tween-20), post-fixed with ice-cold methanol for 3

min, rinsed at least four times with PBST and blocked in PBST/10% fetal bovine serum (FBS) for 1 h at room temperature. They were then incubated in PBST/2% FBS with 1:200 diluted mouse anti-tyrosine hydroxylase antibody (Chemicon, MAB318) for at least 36 h at 4 °C, washed three times for 1 h in PBST at 4 °C, and incubated at 4 °C overnight with 1:500 diluted goat anti-mouse IgG conjugated to Alexa 488 (Invitrogen) in PBST/2% FBS. After three 1-h washes in PBST, the brains were mounted in 80% glycerol/0.1 M Tris-HCl pH 7.5. TH-positive neurons of the DM and DL1 clusters were counted in about thirty 2 micron-thick confocal brain sections per fly.

Statistical analysis

JMP software (SAS Institute) was used for statistical evaluations. Lifespan (survival) assays were analyzed with the Kaplan–Meier log-rank statistical test. Brain ROS levels were compared by one-way ANOVA. For all other experiments, data were compared using the Student's t-test. Results are expressed as mean ± standard deviation.

Results

Increased vulnerability of *parkin*-deficient *Drosophila* to environmental pesticides and mitochondrial toxins associated with Parkinson's disease

For our studies we used the *park25/25* flies in which 75% of the Parkin coding sequence, including the start codon, had been removed by imprecise excision of a P-element insertion at the 5' end of the fly *parkin* gene (Greene et al., 2003). The deletion is thus a *parkin* null allele. As controls, we used *park25/+* (heterozygous mutant *parkin*) flies. To investigate whether *parkin* deficiency increases the susceptibility to environmental substances that are known to cause oxidative stress and have been associated with PD ([Abbott et al., 2003], [Dauer and Przedborski, 2003], [Langston et al., 1983] and [Petrovitch et al., 2002]), we compared the survival of *park25/25* and *park25/+* flies exposed to rotenone, MPP+ and paraquat. Flies were raised on food supplemented with 200 μM rotenone, 25 mM MPP+ or 10 mM paraquat and their survival was monitored daily (Fig. 1). The toxin concentrations we used were in the range

reported for the same pesticides in *Drosophila* in previous publications, namely 2–40 mM for paraquat ([Jones et al., 2009], [Lee et al., 2009], [Pesah et al., 2004], [Schriner et al., 2009] and [Wang et al., 2008]) and 25–500 μM for rotenone ([Coulom and Birman, 2004], [Hosamani and Muralidhara, 2009], [Wang et al., 2007] and [Wang et al., 2008]). For MPP+, we found no reference for *Drosophila* and choose a concentration that is at least 25 times higher than required to induce significant cell death in mammalian neuroblastoma cells (Bando et al., 2005). *park25/25* flies were more sensitive to and showed significantly shortened lifespan after treatment with all three toxins, when compared to either *park25/+* ($p < 0.001$) or *yw* ($p < 0.001$) controls (Fig. 1A–C). In contrast, *park25/+* flies were not more vulnerable to the toxins than *yw* control flies. All toxins further reduced the lifespan of *park25/25 Drosophila* ($p < 0.01$), showing that the experiment was able to detect an effect of the toxins on the *parkin*-deficient background. Unexpectedly, *park25/+* flies displayed increased lifespan compared to *yw* control flies on MPP+, an observation that we have not investigated further and for which we have no explanation at present. We believe it is unlikely that *park25/+* flies developed mitochondrial resistance to MPP+, because both MPP+ and rotenone are potent inhibitors of the mitochondrial complex I, but *park25/+* flies are not resistant to rotenone (Fig. 1A).

Lack of dopamine neuron death in Parkin-deficient flies exposed to environmental toxins

Although dopamine neurons of mice, and most of the dopamine neuron clusters in flies, survive in the absence of Parkin ([Goldberg et al., 2003], [Greene et al., 2003], [Itier et al., 2003], [Pesah et al., 2004] and [Whitworth et al., 2005]), mitochondrial abnormalities and the upregulation of components of the oxidative stress response in *parkin*-deficient *Drosophila* suggested that Parkin generally functions to protect mitochondria and cells against oxidative stress ([Greene et al., 2005], [Greene et al., 2003] and Palacino et al., 2004 J.J. Palacino et al., Mitochondrial dysfunction and oxidative damage in *parkin*-deficient mice (Palacino et al., 2004]). Given the reduced lifespan of *park25/25* flies exposed to pesticides and MPP+ (Fig. 1), we tested whether

dopamine neurons of *parkin* null flies are more sensitive to oxidative stress and mitochondrial complex I inhibition evoked by the same toxins. Dopamine-synthesizing neurons in whole *Drosophila* brains were identified with an antibody against tyrosine hydroxylase (TH). To maximize the chance of detecting a degenerative process, brains of survivors were analyzed when at least 50% of the *parkin*-deficient flies had died. Owing to the small size of the fly brain, TH-positive neurons could be directly counted in serial optical sections of whole-mount fly brains using confocal laser microscopy (Fig. 2A and B). *Drosophila* dopamine neurons are organized in several distinct clusters. We quantified the number of dopamine neurons of the protocerebral posterior lateral (PPL1, also named DL1) and protocerebral posterior medial (PPM, also named DM) clusters (Whitworth et al., 2005). However, no differences in the numbers of TH-positive neurons between wild type and *parkin* null flies exposed to the various toxins were observed (Fig. 2C and D).

Sequestration of redox-active trace metals prolongs the lifespan of *parkin* mutant flies

As discussed above, trace metals can be toxic when insufficiently sequestered and have been implicated in several neurodegenerative disorders ([Friedman et al., 2007], [Rhodes and Ritz, 2008], [Rossi et al., 2004], [Sayre et al., 1999], [Zatta et al., 2009] and [Zecca et al., 2004]). To investigate whether *parkin* deficiency may sensitize flies to endogenous iron and copper toxicity, *park25/25 Drosophila* were raised on food supplemented with chelators of copper and iron. We used the copper-specific chelator bathocuproine disulfonate (BCS) and bathophenanthroline sulfonated sodium salt (BPS), which preferentially sequesters iron and to a lesser degree copper. Various concentrations of the chelators were tested. We found that both 0.1 mM BPS and 0.3 mM BCS significantly prolonged the median lifespan of *park25/25* flies, which was 5 days on normal food (Fig. 3A). In addition, the combination of BPS and BCS extended the median lifespan even more to 19 days (Fig. 3A). In contrast, metal chelation did not increase the lifespan of *park25/+* flies observed up to 50 days of age (data not shown). Because increased longevity has been associated with calorie restriction in several species including

Drosophila (Guarente and Picard, 2005), we wanted to exclude the possibility that the flies consumed reduced amounts of food containing metal chelators. For this purpose we carried out the adult gustatory assay and quantified for food uptake. This assay results in red abdomen coloration due to the chemical acid-red mixed into the food ([Bahadorani et al., 2008] and [Orgad et al., 1998]). As shown in Fig. 3B and C, there was no difference in the amount of food ingested by *park25/25* and *park25/+* flies grown on metal chelator-supplemented food, compared to normal food. This demonstrates that *parkin* mutant flies were not repelled from eating food containing metal chelators. Therefore, our results show that the increased lifespan of *parkin* mutant flies is due to metal chelation and suggest that *parkin* mutants are more sensitive to endogenous levels of the redox-active metals iron and copper, which have been implicated in PD ([Desai and Kaler, 2008], [Friedman et al., 2007], [Hirsch, 2006], [Wright et al., 2009] and [Zecca et al., 2004]). The mechanisms by which iron and copper chelators increased the lifespan of *parkin*-deficient flies are unknown, but possibly include reduced oxidative stress, as both iron and copper have the ability to accept and donate electrons. This can lead to formation of ROS and oxidative modification of proteins, lipids and other cell constituents ([Barnham and Bush, 2008] and [Halliwell and Gutteridge, 1990]). Consistent with this idea, heads of *parkin*-deficient flies raised on chelator-supplemented food showed a reduced level of ROS measured by 2',7'-dichlorofluorescin diacetate (DCF-DA), a cell-permeable non-fluorescent dye that turns highly fluorescent upon oxidation within cells (Fig. 3D). DCF fluorescence of isolated *park25/25* brains, as observed in the confocal microscope, was also significantly lower in presence of metal chelators (Fig. 3D, inset). To further probe an interaction of iron and copper with *parkin* deficiency, we exposed *park25/25* and *park25/+* flies to increased levels of iron and copper. Iron slightly but significantly reduced the lifespan of *park25/25* flies ($p < 0.01$) without exerting an effect on *park25/+* flies (Fig. 3E). In contrast, increasing the copper concentration in the food failed to shorten the lifespan of either genotype of flies (Fig. 3F). It is possible that uptake of iron and copper is subject to different regulation, and that excess copper in the food not necessarily results in increased copper uptake through the copper transporter (see Discussion).

Lack of Parkin aggravates the rough eye phenotype caused by overexpression of the Ctr1B copper importer

High affinity copper transporters are present in all eukaryotes including *Drosophila*, which encodes three members (Ctr1A, B and C) ([Dancis et al., 1994], Lee et al., 2000 J. Lee et al., Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant ([Lee et al., 2000], [Selvaraj et al., 2005], [Turski and Thiele, 2009] and [Zhou et al., 2003]). Ectopic overexpression of the *Drosophila* Ctr1B gene results in copper hypersensitivity leading to death when flies are grown on food with normal copper content (Selvaraj et al., 2005). To study whether *parkin* deficiency may sensitize to copper-mediated cell death in the eye, we overexpressed Ctr1B under the control of the eye-specific GMR promoter in *park25/+* and *park25/25 Drosophila*. Control GMR-Ctr1B transgenic flies showed normal eyes when grown on regular food (Fig. 4A), but developed a rough eye phenotype in presence of 20 μM copper (Fig. 4B). *park25/25 Drosophila* also had normal eyes (Fig. 4C). In contrast, GMR-driven overexpression of Ctr1B in *park25/25* flies resulted in a rough eye phenotype, even on regular food (Fig. 4D). This phenotype was alleviated by copper scarcity and exacerbated by increased copper levels (data not shown). Importantly, the rough eye phenotype was not observed when Ctr1B was overexpressed in the eyes of *park25/+* flies (Fig. 4E). These results show that loss of Parkin and Ctr1B overexpression synergize in the induction of the rough eye phenotype, and suggest that *parkin* deficiency increases the vulnerability to increased copper import through Ctr1B. To investigate whether the phenotype was indeed specific for copper, park25/25 flies overexpressing Ctr1B were raised on food supplemented with 20 μM or 50 μM silver nitrate (AgNO3). Silver effectively competes with and inhibits copper import through the human transporter hCtr1 (Lee et al., 2002) but, in contrast to copper, does not promote oxidative stress by engaging in cellular redox reactions. Silver failed to cause a rough eye phenotype in *park25/25*

flies overexpressing Ctr1B (Fig. 4F–G), showing that this phenotype was specific to copper and likely depends on copper-mediated oxidative stress.

Beneficial effects of antioxidants and SOD1 overexpression on *parkin*-deficient flies

Because the studies described above suggested that reduced ROS production may, at least in part, be responsible for the increased lifespan of *parkin*-deficient flies raised on metal chelator-supplemented food (Fig. 3A and D), we further investigated the role of oxidative stress in the reduced longevity of *park25/25* flies and the effect of combined anti-oxidant and metal chelator treatment on the survival of *park25/25* flies. Cytosolic Cu/Zn superoxide dismutase (SOD1), catalase and glutathione reductase provide the first line of defense against ROS. In addition, the glutathione precursor N-acetylcysteine (NAC), which can be taken up by cells, reduces oxidative stress through its ability to scavenge free radicals. Ascorbic acid (vitamin C) is the most important water-soluble antioxidant and protects cellular DNA and low-density lipoproteins from oxidative damage by quenching free radicals (Duarte and Lunec, 2005). Both 15 mM NAC (Fig. 5A) and 100 mM ascorbic acid (Fig. 5B) significantly increased the median lifespan of *park25/25* flies from 5 days to 14 days (NAC) and 10 days (ascorbic acid). However, higher concentrations of ascorbic acid were ineffective and even shortened lifespan. Although this was unexpected, studies in humans have shown that optimal dosing of vitamin C is critical to achieve beneficial effects (Padayatty et al., 2003). Flies grown on food containing both NAC and metal chelators had a longer maximum lifespan (>38 days) than either flies grown on NAC-supplemented food alone (22 days) or metal chelators alone (30 days). However, the median lifespan of flies treated with NAC and metal chelators (15 days) was similar to flies treated with NAC alone (14 days), suggesting that the combination of both treatments provides no further benefit on overall survival (although it extends maximum lifespan). Collectively, these results suggest that the lifespan-extending effect of metal chelation is at least partially mediated through the reduction of oxidative stress. Next, we compared the survival of *parkin*-deficient flies with or without overexpression of SOD1 in

mesoderm-derived tissues. In this experiment, survival was monitored at the four specific ages indicated in Fig. 5C. SOD1-expressing *park25/25* flies up to 4 days of age did not show a difference in survival compared to non-transgenic *park25/25* flies (Fig. 5C). However, at 11 days of age only 10% of the *park25/25* flies were alive compared to 80% of the *park25/25* flies overexpressing SOD1. None of the *park25/25* flies survived to 17 days at which time point 80% of the SOD-1 expressing *park25/25* animals were still alive (Fig. 5C). In conclusion, both non-enzymatic and enzymatic reduction of oxidative stress and ROS led to increased survival of *park25/25* flies. The effect of SOD1 was particularly strong, perhaps because thetwi-GAL4 driver results in enhanced SOD1 expression in flight muscles ([Estrada et al., 2007] and [Greig and Akam, 1993]), which underwent apoptotic degeneration in *parkin*-deficient *Drosophila* (Greene et al., 2003).

Loss of *parkin* leads to increased susceptibility to hypoxia and heat shock

It has been proposed that Parkin functions as a multipurpose neuroprotective agent in the defense against oxidative stress and the accumulation of specific Parkin substrates ([Feany and Pallanck, 2003] and [Moore, 2006]). In addition, *parkin* mutant flies were reported to be more sensitive to cold stress (Pesah et al., 2004). A recent study identified an E3 ligase complex comprised of Parkin and two other recessive PD-associated proteins, PINK1 and DJ-1 (PPD complex), whose function is to degrade unfolded and misfolded proteins (Xiong et al., 2009). In order to analyze whether Parkin may also protect against non-oxidative stressors and conditions that induce global protein denaturation and aggregation, we exposed *park25/25* and *park25/+* flies to transient hypoxia and heat shock. The cellular response to hypoxia is conserved in Drosophila (Lavista-Llanos et al., 2002) and the recovery from oxygen deprivation is significantly delayed by mutations in hypnos genes (Haddad et al., 1997), one of which encodes an RNA-directed adenosine deaminase (ADAR) (Ma et al., 2001). ADAR is known to edit the transcripts of at least three fly ion channels, suggesting that regulation of specific ion channels might be important for recovery from hypoxia (Ma et al., 2001). Interestingly, the recovery of *park25/25* flies from a 7-min exposure to 0.5%

oxygen in a hypoxia chamber was also significantly delayed when compared to *park25/+* control flies (Fig. 6A). In addition, a heat shock for 30 or 60 min at 38 °C resulted in dramatically increased mortality in the absence of Parkin, with only 15% and 7% of the *park25/25* flies surviving, while at least 95% of *park25/+* control flies survived the same treatments (Fig. 6B). These results suggest that the protective function of Parkin extends to non-oxidative stressors and conditions that impair global protein homeostasis.

Discussion

Here, we addressed whether the lack of Parkin sensitizes flies to environmental pesticides, mitochondrial toxins and metals that have been implicated as risk factors in the etiology of Parkinson's disease. Earlier studies in *Drosophila* had shown that *parkin* ablation increased the vulnerability of flies to paraquat (Pesah et al., 2004), but other toxins and metals have not been studied previously. Our experiments confirm the reduced lifespan after exposure to paraquat and show that Parkin-deficient flies are also more sensitive to the mitochondrial complex I inhibitors rotenone and MPP+. In rats, chronic and systemic exposure to rotenone reproduces features of PD, including nigrostriatal degeneration and α-synuclein-positive cytosolic inclusions ([Betarbet et al., 2000] and [Sherer et al., 2003]). *Drosophila* expressing mutant forms of LRRK2 exhibited late-onset dopamine neuron loss that was aggravated by rotenone (Ng et al., 2009). Interestingly, co-expression of human Parkin conferred significant protection against rotenone-induced dopamine neuron loss in LRRK2 G2019S transgenic flies, suggesting that Parkin reversed the increased rotenone sensitivity caused by mutant LRRK2 (Ng et al., 2009). MPP+ is the toxic metabolite of MPTP, which caused an acute and permanent state of Parkinsonism in humans ([Langston et al., 1983] and [Langston and Ballard, 1983]). This discovery subsequently led to the development of MPTP-based PD models in monkeys and mice ([Burns et al., 1983] and [Heikkila et al., 1984]). While both rotenone and MPP+ significantly shortened the lifespan of *park25/25 Drosophila*, we failed to detect an adverse effect of these chemicals on the survival of dopamine-synthesizing (tyrosine hydroxylase-positive) neurons in these flies. The same

was true for paraquat that is known to produce nigrostriatal dysfunction and degenerative changes reminiscent of PD in mice ([Brooks et al., 1999], [Manning-Bog et al., 2002] and [McCormack et al., 2002]). Although no loss of TH-positive neurons was observed in *park25/25* flies, we cannot exclude the possibility that dopamine neuron death may occur after longer toxin exposure in *parkin* mutant flies. Limited dopamine neuron death restricted to the PPL1 cluster has been reported in 20-day-old *parkin* mutant flies (*park25* allele) (Whitworth et al., 2005). However, the original characterization of the same flies showed that dopamine neuron numbers were normal, even in 30-day-old *Drosophila* (Greene et al., 2003). In addition, although we used the same flies, the maximum lifespan of park25/25 flies was only 10–13 days in different experiments in our hands (see Figs. 1C, 3A, E, 5A–B), preventing us from analyzing dopamine neuron numbers at later ages. Alternatively, feeding the toxins may not result in their accumulation in the brain at concentrations sufficiently high to affect neurons. In this regard, it is noteworthy that flies with a knockout of DJ-1, another gene associated with Parkinsonism, also displayed a shortened lifespan but no dopamine neuron death when exposed to paraquat and rotenone ([Menzies et al., 2005] and [Meulener et al., 2005]). Collectively, these results show that the dopaminergic system of flies is largely insensitive to paraquat, rotenone and MPP+ (at least under the conditions used here), but that lifespan determination is a feasible strategy to assess the effects of a specific gene ablation on susceptibility to PD-associated toxins and other factors. Therefore, in all subsequent studies we focused on the survival of *parkin*-deficient flies compared to *park25/+ Drosophila* to determine whether a given treatment affected the flies in a *parkin*-dependent manner.

Iron and copper have been implicated in the pathogenesis of various neurodegenerative disorders ([Chinta and Andersen, 2008], [Desai and Kaler, 2008], [Rhodes and Ritz, 2008], [Waggoner et al., 1999], [Zatta et al., 2009] and [Zecca et al., 2004]). The total iron content is increased in the substantia nigra in PD, and a shift in the Fe^{3+}/Fe^{2+} ratio with a higher level of reactive labile Fe^{2+} may contribute to increased oxidative stress in PD (Berg and Hochstrasser, 2006). In our experiments, we found that *park25/25* but not

park25/+ flies showed decreased lifespan when grown on iron-supplemented food. Moreover, addition of the iron-chelating agent BPS to food containing normal levels of iron increased the lifespan of *parkin*-deficient flies. Likewise, mixing the copper-chelating agent BCS into the food significantly increased the lifespan of *park25/25* flies, and the combination of both BCS and BPS extended lifespan even more. In contrast, *park25/+* flies showed similar survival curves up to 50 days of age when grown on either normal or chelator (BPS + BCS) -supplemented food (data not shown), indicating that sequestration of free iron and copper benefited flies only in the absence of Parkin. Given that sequestration of copper extended the lifespan of *park25/25 Drosophila,* we were surprised to find that increased concentrations of copper in the food did not impair survival of *parkin*-deficient flies. It is conceivable that the uptake of copper is tightly regulated and not proportional to the amount of copper added to the food. In fact, copper uptake by the human Ctr1transporter (hCtr1) was saturated at about 25 μM copper (Lee et al., 2002), a concentration that is 20 times lower than we used in the copper feeding experiment (Fig. 3E). In contrast, overexpression of the copper transporter Ctr1B in the eye of *park25/25* flies resulted in a rough eye phenotype even on food with normal copper concentration, while this phenotype was not observed in *park25/+* controls. Because it has been shown that overexpression of hCtr1 increases copper uptake even at low copper concentrations (Lee et al., 2002), these results suggest that the uptake, rather than availability of copper in the food, may be limiting for toxicity in *park25/25* flies. Compared to *park25/+* control flies, *park25/25* animals showed significantly increased levels of ROS in the head/brain, as measured by DCF fluorescence. Rearing *park25/25* flies on food containing iron- and copper-chelating agents decreased the ROS levels, suggesting that reduction of oxidative stress through sequestration of redox-active iron and copper may be responsible for the lifespan extension observed under these conditions. In agreement with this idea, transcriptional profiling of *Drosophila parkin* mutants revealed that genes of the oxidative stress response are up-regulated (Greene et al., 2005). Furthermore, the phenotype of *parkin*-deficient flies was exacerbated by loss-of-function alleles in genes that limit oxidative damage, such as glutathione S-transferase S1 (GstS1), thioredoxin (Trx) and Rlu-A1,

which is involved in the synthesis of the anti-oxidant vitamin B2 (Greene et al., 2005). Conversely, increased glutathione S-transferase activity rescued neuron loss in a different line of *parkin* mutant flies (Whitworth et al., 2005). Finally, our own studies described here corroborate that oxidative stress is responsible for the reduced longevity of *park25/25* flies. Both N-acetylcysteine and ascorbic acid (vitamin C) supplementation significantly increased the lifespan of *park25/25* flies. In addition, overexpression of SOD1 using the mesoderm-specific driver twi-GAL4 dramatically prolonged the survival of *parkin* mutant flies, possibly by enhancing SOD1 expression in muscle tissue, which was affected by apoptotic degeneration in *park25/25* flies (Greene et al., 2003). Combining N-acetyl cysteine with metal chelators extended the maximum but not the median lifespan of *park25/25* flies, suggesting that metal sequestration extends lifespan, at least in part, by reducing oxidative stress. This is consistent with the ability of the iron- and copper-chelating agents to reduce ROS levels in the brain of *parkin*-deficient flies (Fig. 3D).

Recent evidence has placed Parkin in a common pathway with PINK1 in the maintenance of mitochondrial function and integrity ([Clark et al., 2006], [Park et al., 2006] and [Yang et al., 2006]). Mitochondrial dysfunction and, in *Drosophila*, degeneration have been observed in the absence of either protein (Bueler, 2009). However, a unique feature of Parkin is its ability to associate with dysfunctional mitochondria and promote their degradation through mitophagy (Narendra et al., 2008), suggesting that impaired removal of defective mitochondria in the absence of Parkin is pathogenic (Narendra et al., 2009). As a consequence, endogenous and exogenous factors that impose additional stress on mitochondria may result in further accumulation of dysfunctional organelles in Parkin-deficient cells, which could increase toxicity. Both rotenone and MPP+ are potent inhibitors of mitochondrial complex I. In addition, iron is an essential component of many mitochondrial proteins (iron–sulfur cluster proteins) that are readily oxidized, and copper-mediated oxidative stress has been linked to mitochondrial dysfunction in neurodegenerative disorders associated with copper imbalance (Rossi et al., 2004). In line with this, treatment of dopaminergic neurons with copper led to

mitochondrial dysfunction, decreased dopamine content and cell death (Shi et al., 2008). Thus, the various toxins and metals tested in this study may further accelerate and enhance mitochondrial dysfunction and degeneration, overwhelming the mitophagy system that is already compromised in *parkin*-deficient flies. To study whether chelator treatment mitigated mitochondrial degeneration in *park25/25* flies, we examined mitochondrial morphology in sections of the indirect flight muscles by electron microscopy. However, we failed to detect a rescue of swollen mitochondria with disintegrated cristae after chelator treatment (data not shown), showing that the life-extending effect of metal chelation is likely not due to improved mitochondrial structure, but rather due to reduction of oxidative stress that may occur downstream of mitochondrial dysfunction in *park25/25 Drosophila*. In this context, it is worth mentioning that food supplementation with zinc, a non-redox-active metal that under most conditions functions as an antioxidant, exerts a beneficial effect on *parkin* mutant flies (Saini and Schaffner, 2010). Future studies with flies expressing mitochondrial-targeted GFP (Hollenbeck and Saxton, 2005) may be interesting to investigate whether metal chelators can improve other aspects of mitochondrial biology, such as transport and dynamics, in *parkin* mutants.

Finally, our studies show that *park25/25* flies are more sensitive to hypoxia and heat shock. Interestingly, the three recessive PD proteins Parkin, PINK1 and DJ-1 associate to form a functional E3 ligase complex (PPD complex) that promotes the degradation of Parkin substrates as well as unfolded and misfolded proteins after heat shock (Xiong et al., 2009). This strongly suggests that the drastically reduced survival of *park25/25* flies after heat shock is due to impaired removal of misfolded and aggregated proteins that may accumulate under these conditions.

Our studies may have therapeutic implications. The extension of lifespan in *park25/25* flies by a combination of different metal-chelating agents hints towards a possible strategy of reducing oxidative stress in familial PD, where carriers of PARK2 (Parkin) mutations could be identified by genetic screening and treatment may be administered early to inhibit or delay dopamine neuron death. PARK2 mutations are by far the most common in recessive

Parkinsonism ([Abbas et al., 1999] and [Gasser, 2005]), accounting for 49–63% of early-onset familial cases ([Lohmann et al., 2003] and [Lucking et al., 2000]). In addition, PARK2 mutations are found in 19% of isolated PD cases with onset before 55 years ([Lohmann et al., 2003] and [Periquet et al., 2003]). Thus, metal sequestration might benefit a significant number of patients. A previous study showed that the orally administered iron chelator clioquinol was well tolerated in mice, where it resulted in a partial protection against MPTP-induced dopamine neuron degeneration in a model of sporadic PD (Kaur et al., 2003). Our experiments did not directly address whether chelators, in addition to reducing ROS, rescued muscle cell apoptosis (Greene et al., 2003) or non-dopaminergic neuronal cell loss that might occur in the brain of *parkin*-deficient flies. Such studies will be important in the future to refine the mechanisms of metal chelator-mediated lifespan extension in *parkin* mutant flies. In addition, studies in "familial PD mice" that show frank dopamine neuron loss (Lu et al., 2009) will be necessary to test whether metal chelation indeed protects against dopamine neuron death caused by Parkin mutations.

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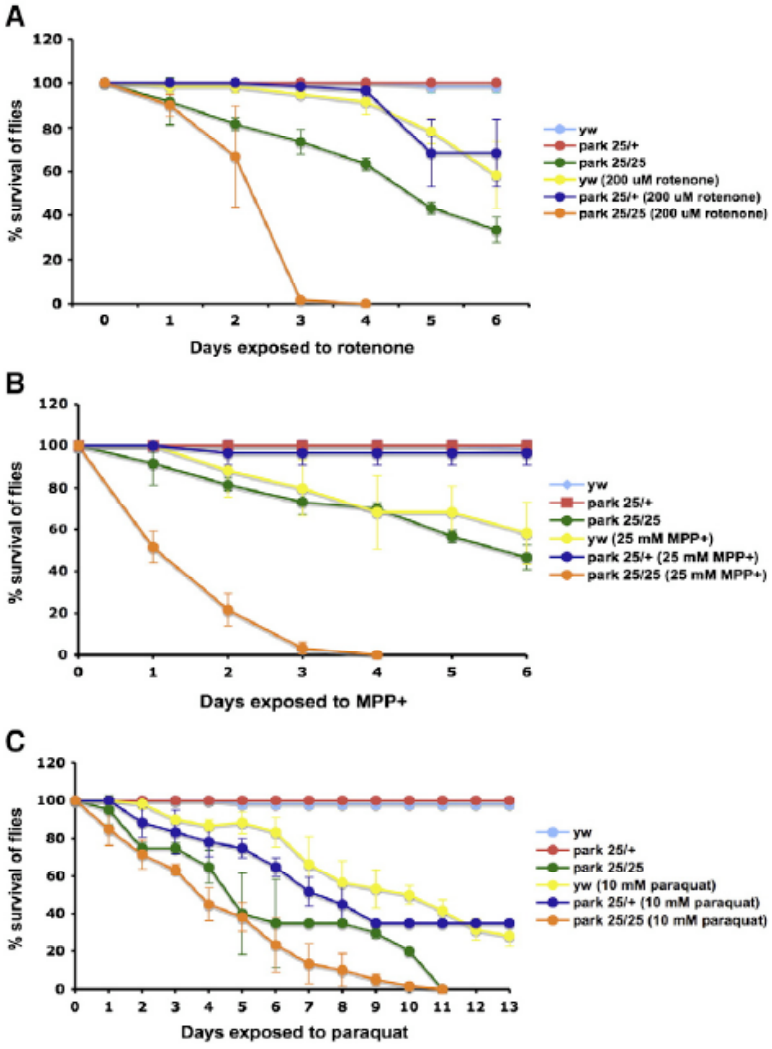


Fig. 1. Lack of Parkin increases sensitivity of flies to environmental toxins and MPP^+. Twenty 1- to 2-day-old flies of each genotype were grown on food supplemented with (A) 200 μM rotenone, (B) 25 mM MPP^+ or (C) 10 mM paraquat and were scored daily for survival. Error bars indicate standard deviation. Data were analyzed using the Kaplan-Meier log-rank statistical test. $park^{25/25}$ vs. $park^{25/+}$ for all toxins tested, $p<0.001$; $park^{25/25}$ normal food vs. $park^{25/25}$ food plus toxin for all toxins tested, $p<0.01$.

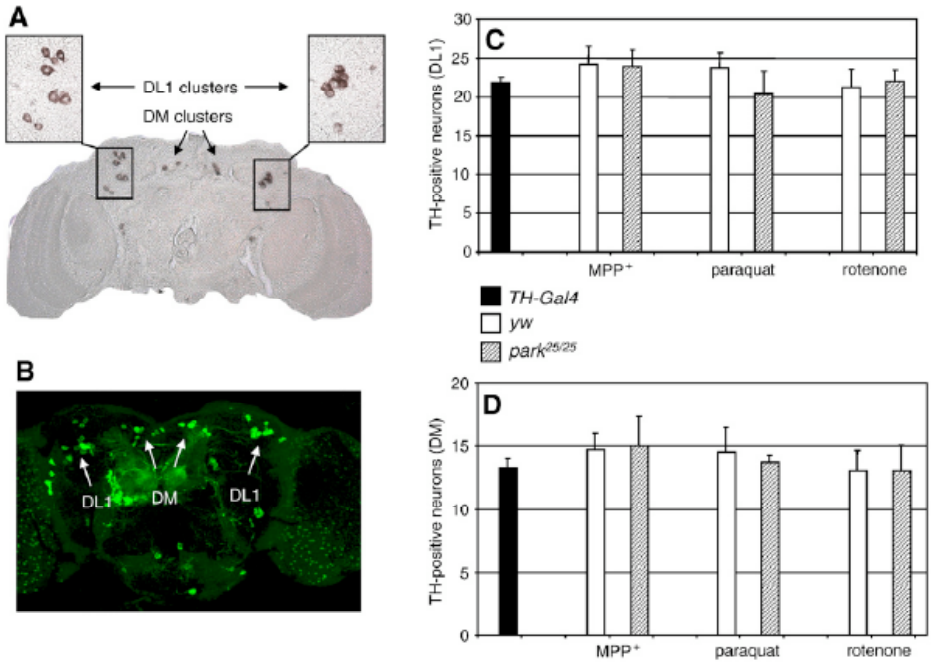


Fig. 2. Normal counts of dopamine neurons in *parkin* mutant flies exposed to environmental toxins. (A) TH-positive neurons of DM/PPM and DL1/PPL1 clusters of the *Drosophila* brain revealed by TH-DAB immunohistochemistry in 5-μM-thick paraffin sections of a wildtype fly brain. (B) TH-positive neurons labeled by fluorescent immunohistochemistry in a whole brain of an adult fly (whole-mount). DL1/PPL1, protocerebral posterior lateral TH neurons; DM/PPM, protocerebral posterior medial TH neurons. (C–D) Lack of dopamine neuron loss in flies exposed to MPP^+, paraquat or rotenone. Dopamine neurons were stained with the anti-TH antibody and quantified by confocal microscopy in whole brains of flies of the various genotypes. Two- to 3-day-old flies were grown on food supplemented with 25 mM MPP^+, 10 mM paraquat or 200 μM rotenone. Neurons of at least seven brains per genotype and toxin were counted for the DL1/PPL1 cluster (C) and DM/PPM cluster (D). Error bars indicate standard deviation.

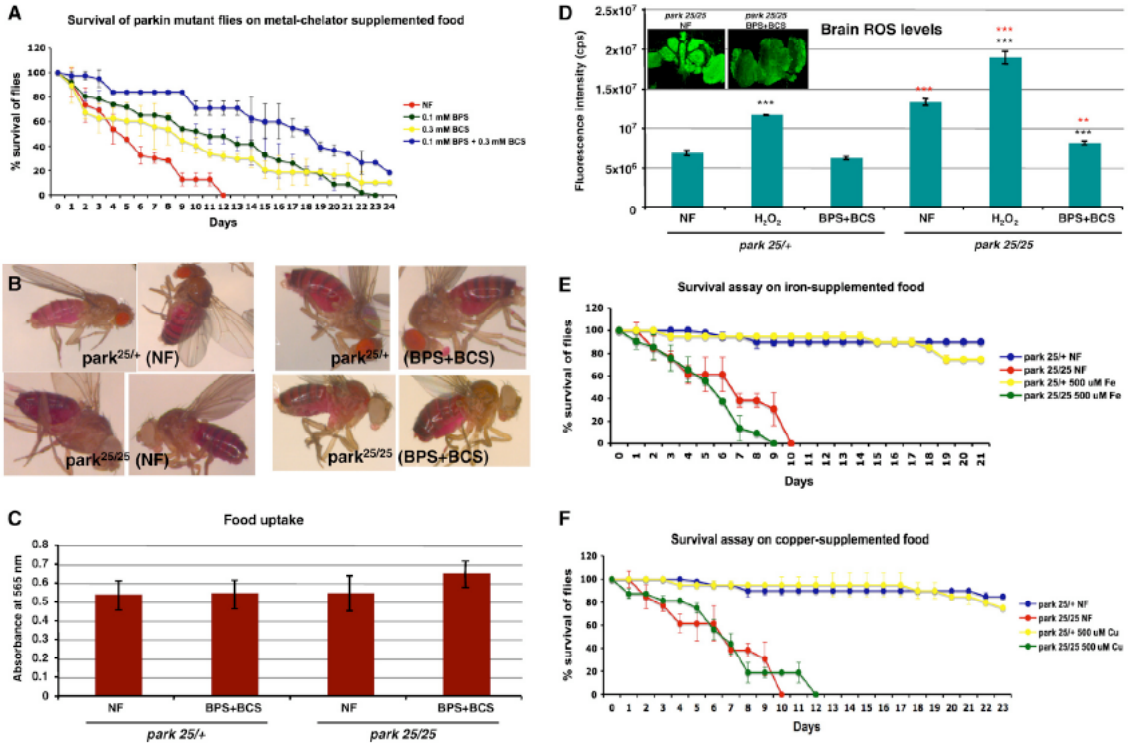


Fig. 3. Lifespan of parkin mutant flies grown on food supplemented with metal-chelating agents and redox-active metals. All experiments were done in triplicate with $n = 20$ flies per genotype and experiment. (A) Flies grown on food containing 0.1 mM of the iron chelator bathophenanthroline sulfonated sodium salt (BPS), 0.3 mM of the copper chelator bathocuproine disulfonate (BCS), or a combination of both metal-chelating agents. $park^{25/25}$ normal food vs. $park^{25/25}$ food plus BCS, $p < 0.01$; $park^{25/25}$ normal food vs. $park^{25/25}$ food plus BPS, $p < 0.01$; $park^{25/25}$ normal food vs. $park^{25/25}$ food plus BCS and BPS, $p < 0.001$. Survival of $park^{25/+}$ control flies was unaffected by the metal chelators up to 50 days of age (data not shown). (B) Qualitative and (C) quantitative gustatory assay, showing that flies consumed comparable amounts of normal and metal chelator-supplemented food (for details, see Materials and methods). Twenty flies per genotype were analyzed. Pictures in panel B were taken at similar but not identical magnification to qualitatively show abdomen redness (D). ROS-dependent DCF fluorescence in the heads and brains of $park^{25/25}$ and $park^{25/+}$ flies, showing that parkin ablation increases oxidative stress, which is reduced after metal sequestration by BPS and BCS. Pools of twenty brains per treatment and genotype were analyzed. ***$p < 0.001$ and **$p < 0.01$ compared to normal food for the same genotype (black stars), or compared to the same treatment between genotypes (red stars). (E) Survival of $park^{25/25}$ and $park^{25/+}$ flies on food containing 500 μM ammonium ferric citrate, $p < 0.01$. (F) Survival of $park^{25/25}$ and $park^{25/+}$ flies on food supplemented with 500 μM Cu(II)-sulfate. p value not significant.

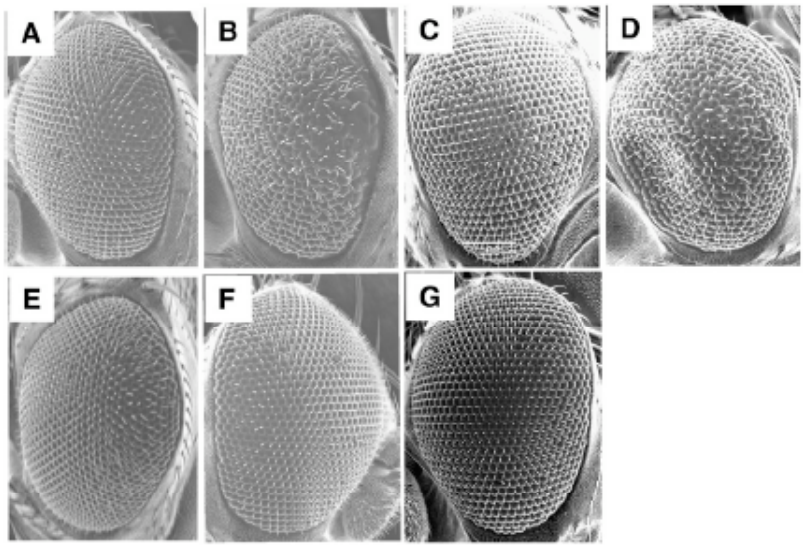


Fig. 4. Overexpression of Ctr1B causes a rough eye phenotype in *park*$^{25/25}$ but not *park*$^{25/+}$ flies. Control *w; GMR-Ctr1B/Cyo* transgenic flies grown on (A) regular food and (B) food supplemented with 20 μM Cu(II)-sulfate. (C) *park*$^{25/25}$ flies. (D) Rough eye phenotype in *w;GMR-Ctr1B/Cyo; park*$^{25/25}$ flies. (E) Normal eye phenotype in *w; GMR-Ctr1B/Cyo; park*$^{25/+}$ flies. Supplementation of food with (F) 20 μM and (G) 50 μM $AgNO_3$ did not cause a rough eye phenotype in *w;GMR-Ctr1B/Cyo; park*$^{25/25}$ flies, showing that the rough eye phenotype in *park*$^{25/25}$ flies overexpressing Ctr1B is copper-dependent (see main text).

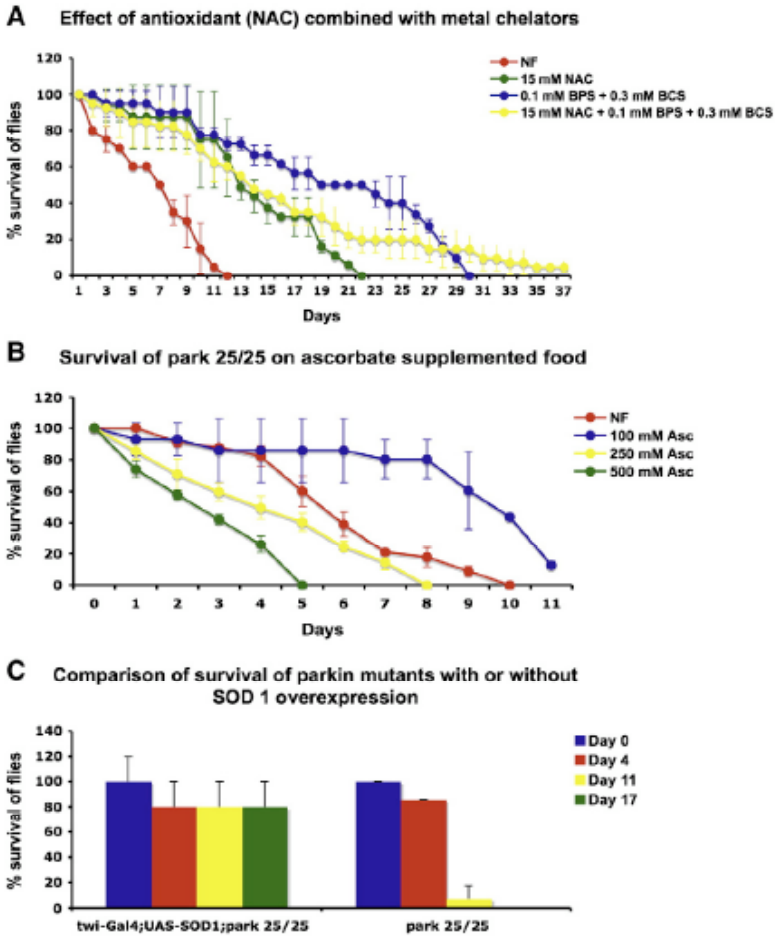
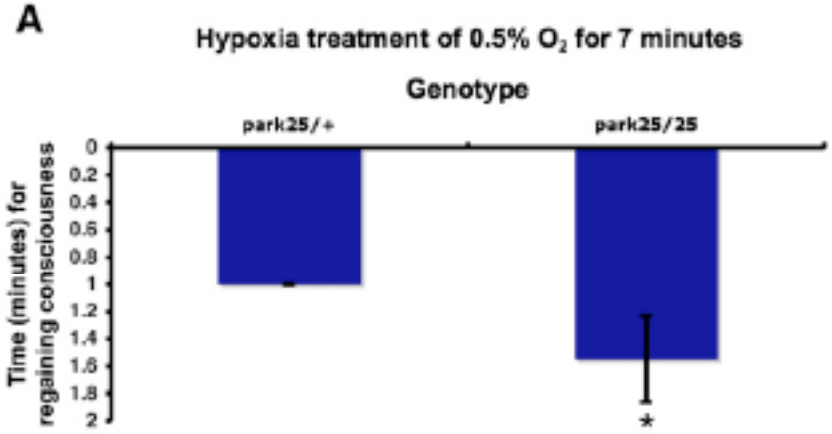


Fig. 5. Effects of antioxidants and SOD1 overexpression on the lifespan of *parkin* mutant flies. (A) $park^{25/25}$ flies grown on food containing 15 mM N-acetylcysteine (NAC), the metal-chelating agents BPS (0.1 mM) and BCS (0.3 mM), or both NAC and metal chelators display increased lifespan compared to $park^{25/25}$ flies grown on regular food. Kaplan–Meier log-rank test, $p<0.01$ for all treatments compared to normal food. (B) Ascorbic acid increased lifespan of $park^{25/25}$ flies at 100 mM (Kaplan–Meier log-rank test, $p<0.01$) but not 250 mM and 500 mM. (C) Overexpression of SOD1 using the *twi-Gal4* driver significantly increased the number of surviving $park^{25/25}$ flies at 11 days and 17 days (paired Student's *t*-test, $p<0.05$), at which time point the experiment was terminated because all $park^{25/25}$ flies had died. Experiments were carried out in triplicate with 20 flies per experiment. Data are expressed as mean ± standard deviation.

A



B	% survival after heat stress	
genotype	38°C, 30'	38°C, 60'
w ; +; park $^{25/+}$	97.5 %	95 %
w; +; park $^{25/25}$	15 %	7 %

Fig. 6. Parkin-deficient flies are more vulnerable to hypoxia and heat shock. (A) *park*$^{25/+}$ and *park*$^{25/25}$ flies (six sets of 20 animals per genotype) were exposed for 7 min to 0.5% oxygen at room temperature (25 °C) in a closed hypoxia chamber, and the time to regain consciousness (ability to move) was recorded. Student's *t*-test, $p<0.001$. (B) *park* $^{25/+}$ and *park*$^{25/25}$ flies ($n=20$ per genotype) were exposed to 38 °C for either 30 min or 60 min and survival was recorded 24 h later. Experiments were done three times. Student's *t*-test, $p<0.01$.

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Parkin mutant in the fly is largely rescued by metal-responsive transcription factor (MTF-1)

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Abstract

The gene for Parkin, an E3 ubiquitin ligase, is mutated in some familial forms of Parkinson's disease, a severe neurodegenerative disorder. A homozygous mutant of the *Drosophila* ortholog of human *parkin* is viable but results in severe motoric impairment including an inability to fly, female and male sterility, and a decreased lifespan. Here we show that a double mutant of the genes for Parkin and the metal-responsive transcription factor MTF-1 is not viable. MTF-1, which is conserved from insects to mammals, is a key regulator of heavy metal homeostasis and detoxification and plays additional roles in other stress conditions, notably oxidative stress. In contrast to the synthetic lethality of the double mutant, elevated expression of MTF-1 dramatically ameliorates the *parkin* mutant phenotype, as evidenced by prolonged lifespan, motoric improvement including short flight episodes, and female fertility. At the cellular level, muscle and mitochondrial structures are substantially improved. A beneficial effect is also seen with a transgene encoding human MTF-1. We propose that Parkin and MTF-1 provide complementary functions in metal homeostasis, oxidative stress and other cellular stress responses.

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Running title: *Parkin* mutant largely rescued by MTF-1
word count for the introduction, results, and discussion sections: 3343
word count for the Materials and methods section: 2084

Abstract

The gene for Parkin, an E3 ubiquitin ligase, is mutated in some familial forms of Parkinson's disease, a severe neurodegenerative disorder. A homozygous mutant of the *Drosophila* ortholog of human *parkin* is viable but results in severe motoric impairment including an inability to fly, female and male sterility, and a decreased lifespan. Here we show that a double mutant of the genes for Parkin and the metal-responsive transcription factor MTF-1 is not viable. MTF-1, which is conserved from insects to mammals, is a key regulator of heavy metal homeostasis and detoxification and plays additional roles in other stress conditions, notably oxidative stress. In contrast to the synthetic lethality of the double mutant, elevated expression of MTF-1 dramatically ameliorates the *parkin* mutant phenotype, as evidenced by prolonged lifespan, motoric improvement including short flight episodes, and female fertility. At the cellular level, muscle and mitochondrial structures are substantially improved. A beneficial effect is also seen with a transgene encoding human MTF-1. We propose that Parkin and MTF-1 provide complementary functions in metal homeostasis, oxidative stress and other cellular stress responses.

Keywords: *Drosophila*/ MTF-1/ metal homeostasis/ *parkin*/ Parkinson's disease

Introduction

Parkinson's disease (hereafter referred to as PD) is the second most prevalent progressive neurodegenerative disorder and the most common age-related movement disorder (10, 13, 43, 59). Many molecular aspects of PD pathogenesis still need to be clarified. Extensive studies point to oxidative stress as a major contributor to the disease (28). Besides the gene for Parkin, an E3 ubiquitin ligase, four other genes: *PINK1*, *DJ1*, *UCHL1* and α-*synuclein* have been implicated in rare, early-onset, familial forms of PD while *LRRK2* is predominantly responsible for late onset PD (20, 57, 70). Much effort has gone into the development of animal models of PD, including models in the fly *Drosophila melanogaster*. In our studies presented here, we use a strain in which the ortholog of the human *parkin* gene has been disrupted by insertion of a P-element transposon into the coding region (24, 48).

In mammals, the proteins PINK1 and Parkin cooperate to ensure proper quality control of mitochondria and Parkin is particularly important for autophagy of faulty mitochondria (reviewed in (8, 73). In agreement with this notion, Parkin deficient flies suffer from mitochondrial malfunction (24, 45, 48), which distorts muscle structure and causes severe locomotor defects and an inability to fly (24, 48). Furthermore, both male and female *parkin* null mutant *Drosophila* are sterile (52), exhibit an increased sensitivity to multiple stresses, including oxidative stress, and have a reduced lifespan (23, 48).

Maintenance of metal homeostasis is an essential requirement for the proper functioning of all organisms. An adequate supply of essential trace metals, like copper and zinc, is important, whereas an excess can be highly toxic. Alterations in copper homeostasis due to mutations in copper transporters cause Wilson's and Menkes disease (11, 31, 65). An imbalance in trace metal levels has also been implicated in neurodegenerative disorders such as Parkinson's and Alzheimer's disease, as well as in senescence processes (42, 53, 54). To investigate the possible interplay of Parkin function with metal homeostasis, we modulated the concentration of the metal responsive transcription factor-1 (MTF-1) in *parkin* mutant *Drosophila.* MTF-1 is conserved in evolution and its homologs have been characterized in humans (7, 36, 44), mice (25, 51, 69), fish (3, 9) and *Drosophila* (17, 60, 75). MTF-1, also referred to

as metal response element binding transcription factor 1, is a zinc-finger protein that regulates transcription of its target genes by binding to DNA sequence motifs known as metal response elements (MREs), which are typically located proximal to the transcription start (12, 27, 32, 41, 64, 68). The majority of MTF-1 preferentially localizes to the cytoplasm in quiescent, non-stressed cells but translocates to the nucleus upon heavy metal load and a number of other stressful conditions (34, 58, 62).

Apart from counteracting the effects of heavy metal load, MTF-1 also induces transcription of metallothionein genes in response to oxidative stress and infection (2, 21, 22). Metallothioneins (MTs), are small, cysteine-rich, metal-binding proteins with a major role in metal homeostasis and detoxification (30, 46). MTs occur in all eukaryotes, as well as in some prokaryotes. Heavy metals like zinc, copper and cadmium are complexed by the cysteine sulfhydryl groups, which can also exert antioxidant function (2, 29). In this context it is noteworthy that in a mouse PD model, dopaminergic (DA) neurons of a MT-knockout mutant are more vulnerable to L-DOPA toxicity than neurons from mice with wild type MT (15, 39). This suggests that MTs play a protective role against DA-quinone induced neurotoxicity. There are more than ten functional metallothionein genes in humans, four in the mouse (50, 71) and at least four in *Drosophila*, termed *MtnA-MtnD* (17, 40). The *Drosophila* MTs are involved to different degrees in the defense against heavy metal stress. MtnA is the most important under copper load, while MtnB preferentially binds cadmium and protects against cadmium intoxication. MtnC and MtnD, despite sharing 67% amino acid identity with MtnB, have only a minor role in protection against heavy metals, at least when MtnA and MtnB are present (16, 18). The *MtnA* and *MtnB* genes (also referred to as *Mtn* and *Mto*, respectively) are differentially regulated during development (61). In addition to metallothioneins, MTF-1 also regulates, in *Drosophila*, the expression of ferritins, the copper importer Ctr1B, the zinc exporter ZnT35C, glutathione S-transferase and an ABC transporter (60, 63, 74).

MTF-1 proteins of human and *Drosophila* are highly similar in their DNA-binding zinc finger region but quite divergent outside of it. Nevertheless, they can largely complement each other in the protection against metal stress (6, 75). A major difference between mammals and *Drosophila* is that metallothionein genes in

mammals are mainly induced by zinc and cadmium whereas in *Drosophila* they are best induced by copper and cadmium (18, 75, 76). Moreover, disruption of the *MTF-1* gene in the mouse results in embryonic lethality (25), which is not the case for fly mutants, which are viable and fertile. However, the fly mutants do display sensitivity to cadmium, zinc and copper load as manifested by a reduced lifespan on heavy-metal supplemented food, and also cannot tolerate copper starvation (17, 60). The *Drosophila* allele $MTF\text{-}1^{140\text{-}1R}$ carrying a 4.1 kb deletion of the coding region has the strongest phenotype and is considered a null mutation (17). We have used this allele for experiments requiring a *MTF-1* loss of function.

A link between cellular heavy metal handling and the *parkin* mutant phenotype was suggested by our previous finding of a partial rescue of *parkin* mutant flies upon zinc supplementation or chelation of redox-active metals (55, 56). In our present study we set out to investigate the interaction between metal homeostasis and Parkin function, or more specifically, MTF-1 and Parkin. We found that *parkin* mutants combined with a knockout of *MTF-1* are not viable, a genetic constellation termed synthetic lethality. *Parkin* mutant *Drosophila* suffer from oxidative stress as a result of heightened ROS production. A strong ubiquitous MTF-1 expression dramatically ameliorates the *parkin* mutant phenotype: our results show that MTF-1 decreases oxidative stress, normalizes concentration of essential trace metals, increases the frequency of development to adulthood, restores female fertility, improves muscle/mitochondrial morphology, locomotion and considerably extends the lifespan of *parkin* mutant flies.

Materials and Methods

Fly food and maintenance

One liter of standard fly food was composed of 55 g cornmeal, 10 g wheat flour, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent Nipagin (15% in ethanol). For the experiments of survival, development, eclosure frequency, ROS measurements, real-time PCR and TEM muscle analysis, several conditions were tested, namely, normal food (NF), NF supplemented with zinc chloride (4 mM) or N-acetylcysteine (15 mM) or bathocuproine disulfonate (BCS, copper chelator) (0.3

mM), and bathophenanthroline sulfonated sodium salt (BPS, iron (copper) chelator) (0.1 mM). All flies were maintained at 25^{o}C on a 12:12 hours light-dark cycle.

Construction of transgenic flies and fly stocks

UAS-MTF-1 flies were generated using the full-length MTF-1 cDNA cloned into the pUAST vector. *Tub-MTF-1* constructs were made by cloning the MTF-1 cDNA under the control of the constitutive α–tubulin promoter. Both the constructs were injected into the *w1118* fly strain along with p(Δ2-3) helper plasmid and transformants were selected based on the eye color (red/orange). The *MTF-1*$^{140\text{-}1R}$ null allele, generated by homologous recombination, was characterized previously (17). The *UAS-MTF-1*, MT (*tub-MtnA*), *MTF-1*$^{140\text{-}1R}$ strains were generated by recombination in our laboratory. The *w; +; tub-MtnA/TM6B (tub-MtnA)*, *w; tub-MTF-1/Cyo;+ (tub-MTF-1)* and *w;Actin-Gal4;UAS-MTF-1/TM6B (Actin-Gal4;UAS-MTF-1)* flies were combined with *w;+;park*$^{25/25}$ (*park*$^{25/25}$) by recombination. Unless specified otherwise, *w;+;park*^{25}*/TM6B,w*$^{+}$ flies (heterozygous *parkin* mutants hereafter referred to as *park*$^{25/+)}$ were used as controls for the *w;+;park*$^{25/25}$ flies (homozygous *parkin* mutants hereafter referred to as *park*$^{25/25}$). *park*$^{25/25}$ is a null mutation of the *Drosophila parkin* gene (24). The TM6B balancer in the control flies was confirmed to have no effect on any of the experiments performed (by removing it).

Lifespan determinations, eclosure frequency and fertility assays

For lifespan experiments, 1-2 day old flies (20 per vial) maintained at 25^{o}C on a 12:12 hours light-dark cycle were examined for each genotype at least in triplicate. Surviving flies were transferred to fresh food vials every 2 days and counted daily. In the experiment with *w;Actin-Gal4;UAS-MTF-1, park* ^{25}*/TM6B* flies, the survivors in 23 parallel independent sets were counted at regular time intervals. In each lifespan assay testing different conditions, the controls of *park*$^{25/+}$ and *park*$^{25/25}$ flies raised on NF were the same. The variations in the average lifespan of control flies in different experiments can be attributed to subtle experimental variations. The metal chelator concentrations selected for ROS determination had no significant effect on feeding behavior of the flies (55). Fertility was assayed by placing single *parkin* mutant males with 3-4 virgin *yw* females and by placing single virgin *parkin* mutant females with two *yw* males. Vials were checked 3-7 days later for the presence of larvae and eclosing adults. For the analysis of eclosure frequency, the number of days allowed

for egg laying and the parent population was the same in all vials of normal food (NF) or zinc (Zn)-supplemented food and progeny flies were counted at the same time.

Behavioral assay (climbing performance and locomotion ability)

The Climbing assay was performed as described (47). Flies of each genotype (*park*$^{25/+}$, *park*$^{25/25}$, *tub-MTF-1,park*$^{25/+}$, *tub-MTF-1, park*$^{25/25}$) were anesthetized with CO_2 and individually counted and placed in food vials 24 hours before the assays were performed to enable a full recovery from the effects of CO_2. Ten flies were placed in an empty 110-by 27-mm vial; a horizontal line was drawn 100 mm above the bottom of the vial and another identical vial was used as a cover to provide more mobile space. After the flies had acclimated for 10 min at room temperature, each genotype was assayed in triplicate for five trials per set per genotype. The procedure involved gently tapping the flies (on a soft surface) down to the bottom of the vial. The flies were given 30 seconds to climb the vial and the number of flies which crossed the 100 mm mark each time were recorded. These values were then averaged and a group mean and standard error was calculated. The mean values of various fly groups were statistically compared using an unpaired Student t-test. The study was repeatedly performed with the same group of flies on every alternate day up to 10 days in an isolation room at 25^{o}C, 60-70% humidity under standard lighting conditions. Preliminary studies indicated no significant difference in the outcome of climbing assays performed in normal light or red light conditions.

Fluorescent protein (EYFP) reporter

The *Drosophila* metallothionein *MtnA* promoter (-446 to +74) was cloned from genomic DNA using the primer pair 5'-CGG GAT CCA GGT ATG GGC TAT TTA GGC C-3' and 5'-GGG ATG GCC CCA AAG GAT CTG-3' in a pCasper4-derived vector carrying EYFP-coding sequence and the SV40 polyA site. Details were reported previously (6, 18). Transgenics of *MtnA-EYFP* combined with *parkin* heterozygous or homozygous knockouts were made. Both fly types were then frozen at the same age and photographs of EYFP expression were taken with a Leica MZ FLIII fluorescence stereomicroscope and a Nikon Coolpix 950 digital camera (Leica, Heidelberg, Germany) at an exposure of 730 ms.

RNA isolation and real-time analysis

Total RNA was purified from adult *Drosophila* tissue using the Nucleospin RNA II protocol (Macherey-Nagel) and eluted in 60 µl of RNase-free water. cDNA was prepared using the Transcriptor High Fidelity cDNA Synthesis kit from Roche. The cDNA obtained was further purified using the AM 1906 Ambion DNA free kit and used for analysis by real-time PCR on the Tecan Genesis 200/8 robot using the Eurogenentec Mesa Green qPCR Mastermix Plus for SYBR assays. The qPCR run was performed on an Applied Biosystem machine (ABI Prism SDS 7900 HT) in a 384 well format with a reaction volume of 10 µl. ΔΔct values were calculated by subtracting the Δct calibrator from the Δct sample, Δct values were calculated by subtracting Δct endogenous control from Δct target gene/calibrator. The normalization strategy used has been described in Vandesompele *et al* (67). All the fold-change values are normalized to respective $park^{25/+}$ values on normal food (NF). The housekeeping genes used were *actin5c*, *TBP* and *GAPDH*. Two sets of primer sequences were used for each of the transcripts quantified: for *parkin*, the first primer set was 5'-AAG ATC ATA TTT GCC GGT AAG GAA-3' and 5'-CGC TTT GCT GAC CCA AGT C-3' which amplify a 73 bp fragment only from the *parkin* heterozygous control flies and the second set was 5'-CAA AGC CCT GTC CAA AAT GC-3' and 5'-GCG CGT GTG CAG ACC AT-3'; for *MTF-1*, the first primer set was 5'-TGT CCG GCT GCG ATA AGG-3' and 5'-GCC ATT GTG CAG ACG AAG GT-3' which amplify a 68 bp fragment from wild-type MTF-1 containing flies and the second set was GCA TTC AAC ACG CGC TAC A-3' and 5'-ACA GTT GAA CGT CTC GCC ATT-3'; for *MtnB*, the first primer set was 5'-TTG CAA GGG TTG TGG AAC AAA-3' and 5'-TGC AGG CGC AGT TGT CC-3' which amplify a 65 bp fragment and the second set was 5'-AAG TCG AGA AAT AGA TAC ATA CAA GAT GGT-3' and 5'-CGC ACT TTT GGG CCG AG-3'; for *foi*, the first primer set was 5'-GTG GCT GCG GGT CTG TTC-3' and 5'-TTT GTG CGA GGC CGA GAT-3' which amplify a 69 bp fragment and the second set was 5'-TGG CGA TGC CCT ACT TCA C-3' and 5'-TGA TCA TCC CCC GCT CAT-3'.

Detection of ROS levels

Fresh dichlorofluorescin diacetate (2,7-DCFH-DA) from Invitrogen – Molecular Probes Cat # C369 was mixed with DMSO to make a 1 mM stock solution (=2,7-

DCFH-DA/DMSO). A 40 µM working solution was prepared in HEPES buffer (30 mM). 50 heads of frozen adult flies of a specific genotype (same age) were removed and collected in an Eppendorf tube. This was done in triplicate for each condition. Each sample was then homogenized using cold protein homogenization buffer (1:3 w/v of 0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, 0.5 mM PMSF protease inhibitor at pH 7.4) and centrifuged for 20 minutes at 4^oC and 20,000 g. Protein content of the supernatant was determined using the Bio Rad diagnostics kit and a final concentration of 0.4 mg/ml was used as the standard for the ROS assay of each genotype tested. The fluorescence intensity (emission acquisition) was monitored for 45 minutes after the sample (20 µl) was kept in a cuvette (1.5 x 1.5 nm) in the fluorimeter immediately after the addition of the DCFH-DA dye (2 µl) to prevent loss of signal due to fading of fluorescence. Excitation of dye was at 485 nm and emission at 520 nm. The curve area of fluorescence intensity which was recorded every 5 minutes (for 45 minutes) in the range of 500-600 nm was integrated and the total area was used for comparison, with the final result obtained in counts per second. The fluorimeter was standardized using 0.05% H_2O_2, a positive ROS generating species.

Dissection of ovaries

Ovaries from female parents were dissected in Grace's insect medium (1X), GIBCO, Invitrogen at room temperature (RT). The dissected ovaries were immediately fixed in 4% paraformaldehyde, 0.2% Triton-X-100 dissolved in Grace's medium for 20 minutes without shaking, The fixative was washed three times with phosphate buffer saline (PBS)+0.5% Triton (PBST) for 10 minutes each. The samples were then labeled for one hour with fluorescently labeled phalloidin (Phalloidin-alexa 568, molecular probes), diluted 1:200 in PBST at RT. This was followed by three washes with PBST for 10 minutes each and a second labeling with Toto (nuclear stain) diluted 1:1000 in PBST at RT. The samples were then washed three times with PBST for 10 minutes each, followed by two washes with PBS for 10 minutes each and then embedded in vectashield (mounting medium) overnight at -20^oC. Ovarioles were dissected from the ovaries and mounted on glass slides. Pictures were taken using the confocal at 20X magnification.

Quantification of metal content

Female flies (*park*$^{25/+}$, *tub-MTF-1, park*$^{25/+}$ and *yw* and *MTF-1* knockout controls) were allowed to lay eggs on normal food or metal-supplemented food (100 μM cadmium sulphate/ 500 μM copper sulphate/ 500 μM ammonium ferric citrate/ 4 mM zinc chloride) for four days and removed afterwards. The resulting progeny were collected at regular intervals and frozen. This procedure was repeated until the required number of 50 flies was obtained in triplicate for each genotype. Each sample set of frozen flies was then subjected to homogenization using cold protein homogenization buffer (0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, 0.5 mM PMSF protease inhibitor at pH 7.4) and the samples were normalized for protein content. A final concentration of 1 mg/ml was prepared by diluting the samples in 0.2M HNO_3 to obtain a total assay volume of 1 ml. A highly sensitivity flame atomic absorption spectrophotometer (FAAS; GTA-120/PSD-120, Varian Australia Pty Ltd, Mulgrave, VIC, Australia) was used to detect the metal content in each genotype assessed. Cd and Zn concentrations were recorded by the same flame. Likewise Cu and Fe concentrations were measured together.

Muscle section and TEM

Dissected thoraces of two-day old anesthetized adult flies (*park*$^{25/+}$, *park*$^{25/25}$ and *Act-Gal4; UAS-MTF-1,park*$^{25/25}$) were kept in ice-cold fixative (2.5% glutaraldehyde/0.1 M sodium cacodylate buffer adjusted to 328 mOsm/l with sucrose, pH 7.4) for 4 hours at 4°C. Postfixation was performed with 1% OsO_4 and 0.1 M sodium cacodylate, pH 7.4 for 2 hours at 4°C and the sample was washed overnight with 0.1 M sodium cacodylate, pH 7.4 before going through a series of progressive dehydration steps in a graded ethanol series of 70/80/96% alcohol for 10 minutes each, followed by three 10 min washes in 100% alcohol and a final 20 min wash in propylene oxide. The sample was treated with a 1:1 propylene oxide: Epon (Epon 812) mix for 2 hours and embedded in Epon overnight at 70^{o}C. Blocks of thoraces were trimmed and semi-thin sections of dorsal longitudinal muscles were stained with Toluidine Blue dye, which labels nucleic acids, hence staining both nuclei and cytoplasm (5). Ultrathin sections of 70 nm thickness were made with an ultramicrotome for the selected sections. For TEM, sections were contrast-stained on the grid first with 2% uranyl acetate, then with 2.5% lead citrate (Reynold's), each of

them for twenty minutes at room temperature. The sections were inspected with a Philips CM100 TEM with GATAN Orius Camera.

Statistical Analysis

JMP software (SAS Institute) was used for statistical evaluations. Lifespan (survival) assays were analyzed with the Kaplan–Meier log-rank statistical test. Brain ROS levels and qPCR results were compared by one-way ANOVA. Results are expressed as mean ± standard deviation.

Results

Synthetic lethality of combined *parkin* and *MTF-1* mutants

The metal-responsive transcription factor (MTF-1) is a key regulator of metal homeostasis in *Drosophila* (17, 60, 75). Based on our findings of a connection between the *parkin* knockout and trace metal status (55), we tested the effect of an *MTF-1* knockout in a *parkin* mutant background. The result was clear cut, in that no surviving double mutant was ever observed in 50 independent crosses. This "synthetic lethality," observed at the pupal stage, was rescued by a cDNA transgene of *MTF-1* driven by the constitutive tubulin enhancer/promoter. In a genetic cross between parents heterozygous for the *parkin* and *MTF-1* recombined deletions (Figure 1), 69 out of a total of 217 progeny (32%) were *parkin* and *MTF-1* homozygous knockouts also expressing an *MTF-1* transgene (statistical expectation, 40%). This rescue by an MTF-1 cDNA transgene confirms the absence of any secondary hits as the cause of the observed lethality. Furthermore, it also excludes the possibility that an intronic open reading frame located within the *MTF-1* gene (D. Steiger, K. Steiner and WS, unpublished) is responsible for the effect and not MTF-1 itself. To find out what condition could overcome the pupal lethality, we maintained the heterozygous *parkin, MTF-1* parent flies on N-acetylcysteine (NAC), which is a precursor to glutathione, an established antioxidant. Since either condition, lack of a functional *parkin* gene or of an *MTF-1* gene, increases reactive oxygen species (ROS) (see below), we reasoned that keeping a lower ROS level by other means might also overcome the synthetic lethality of the double mutants. After testing different concentrations of NAC an optimal supplement of 15 mM was chosen for the main experiment. Indeed, raising

the progeny under this condition resulted in a substantial rescue of the synthetic lethality: of the total progeny, 19% was homozygous *parkin* and *MTF-1* double mutant (statistical expectation, 25%) (Table 1A). Other antioxidants tested included ascorbate, zinc, and metal chelators of copper and iron (BCS and BPS, respectively) which we had shown before to positively influence the *parkin* mutant phenotype (55, 56). However, none of these was able to rescue synthetic lethality.

MTF-1 overexpression rescues the lifespan and the low eclosing frequency of *parkin* mutants

We examined the lifespan of *Drosophila MTF-1*-overexpressing transgenic lines (*tub-MTF-1* and *Act-Gal4; UAS-MTF-1*) in a $park^{25/25}$ background. These experiments illustrated that an elevated expression of MTF-1 from the ubiquitously active *tubulin* enhancer/promoter prolonged the lifespan of *parkin* mutants significantly, from a median of 7 days for the mutants alone to 21 days (Figure 2A). In this experiment, the maximal lifespan was extended from 12 to 41 days by the *MTF-1* transgene (Figure 2A). The stronger combination with *actin-Gal4* driving *UAS-MTF-1* revealed a similar effect: in an experiment with 23 independent replicas, 10% of the mutant animals were still alive at day 34 (Figure 2B). Overexpression of MTF-1 in control and wild type flies ($park^{25/+}$ or $park^{+/+}$) did not increase their normal lifespan (data not shown). Also, in an independent study, MTF-1 overexpression did not extend lifespan of flies kept on standard food (4). Elevated MTF-1 expression not only prolonged the lifespan of *parkin* mutant adult flies but also enhanced survival during development. In a genetic cross involving *parkin* heterozygous parents, only 2.5% of the eclosing progeny flies were homozygous *parkin* mutants (statistical expectation, 25%). In comparison, the same cross but also expressing an MTF-1 transgene resulted in a 15% eclosure frequency (Table IB).

Elevated expression of MTF-1 rescues female fertility and fecundity of *parkin* mutant flies

Strikingly, female fertility and fecundity was completely rescued by *MTF-1*. When crossed with wild type males, *parkin* mutant females with the tubulin-driven *MTF-1* transgene produced the same number of progeny as a cross of wild type males and

females. *Drosophila* gonad formation requires a complex morphogenetic process (35, 37). As in the majority of metazoans, *Drosophila* oogenesis occurs within the ovarian follicles in which germline cells develop in close proximity to specialized somatic cells (Figure 3A-A''). Parkin mutant females lack the proper spatio-temporal development in the germarium and thus have stunted ovaries with few mature oocytes, which fail to get fertilized (Figure 3B-B'''). The restoration of female fertility by strong MTF-1 expression was also evident at the morphological level: dissected ovaries showed a normalized structure with follicles formed in the germarium and mature stages in the posterior regions of the ovariole, with several oocytes ready for fertilization (Figure 3C-C''). In contrast, the sterility phenotype of *parkin* mutant males which is due to defective spermatogenesis at the individualization step (24) was not rescued. This suggests that Parkin is particularly important for male fertility.

Improved locomotion and rescued mitochondrial/myofibrillar morphology of *parkin* mutants with enhanced MTF-1 expression

Strong MTF-1 expression dramatically improved the climbing ability of *parkin* mutant flies (Figure 4). What is more, they generally moved around fast, responded by running away when physically perturbed, jumped and occasionally displayed short flight episodes (data not shown). To investigate the effect of elevated MTF-1 expression on muscle morphology we examined the ultrastructure of the indirect flight muscle in heterozygous control flies, *parkin* mutants and the MTF-1 transgenic flies. Cross-thoracic sections of control adults analyzed by transmission electron microscopy (TEM) revealed well organized muscle fibres in parallel stripes with a regular M- and Z-line banding pattern and darkly stained, electron-dense mitochondria with regularly packed cristae (Figure 5A-C). In contrast, age-matched *parkin* mutants had abnormal muscle structure with large vacuoles, a reduced muscle content with mostly irregular arrangement and enlarged mitochondria with disintegrated cristae (Figure 5D-F). The MTF-1 overexpressing *parkin* mutant flies displayed a clear rescue effect in that muscle fibre structure was more regular with less prominent vacuoles; moreover, mitochondria had more densely packed cristae with considerably lesser signs of disintegration in comparison to *parkin* mutants (Figure 5G-I).

MTF-1 dependent expression of metallothioneins is higher in *parkin* mutant *Drosophila*

Owing to their high cysteine content, metallothioneins can act as antioxidants, in addition to their obvious role as metal chelators (33, 38). Basal and induced levels of metallothionein expression depend on the transcription factor MTF-1 (17, 51, 74). To find out whether metallothionein genes could be induced by MTF-1 in a *parkin* mutant background, we used a reporter line in which a yellow fluorescent protein (EYFP) is driven by the promoter of *MtnA*, the most highly expressed *Drosophila* metallothionein gene. Compared to heterozygous controls, even in the absence of any heavy metal load the basal expression of this reporter was increased, likely due to elevated oxidative stress in the *parkin* mutant flies (Figure 6C and D). This metal-independent upregulation of the *MtnA* promoter, was strictly dependent on MTF-1, since no trace of fluorescence could be detected in flies lacking MTF-1 (data not shown). RT-PCR of *parkin* mutants also revealed elevated transcript levels of *MTF-1* (2-fold) (Figure 7A) and the embryo-enriched metallothionein *MtnB* (6-fold) (Figure 7B) compared to $park^{25/+}$ flies. MTF-1 overexpression in the $park^{25/25}$ flies was achieved from the tubulin promoter or indirectly with the stronger UAS-Act-Gal4 system, both induced a more than 200-fold increase in *MtnB* transcripts (Figure 7B). Conversely, the level of *parkin* transcripts was increased in the *MTF-1* knockout flies (Figure 7C). This pattern of regulation can be explained by a partial redundancy of Parkin and MTF-1 where one is upregulated to compensate for the loss of the other. The synthetic lethality of a combined knockout of *parkin* and *MTF-1* genes mentioned above is in line with this hypothesis. Another experiment revealed that the transcript levels of the zinc importer *foi* (37) in *parkin* mutants were enhanced 3-fold by elevated MTF-1 expression, which may contribute to the normalized structure of ovaries and rescued fertility of female *parkin* mutants (Figure 7D). This hypothesis is further supported by the increased level of zinc ions found in tub-MTF-1; $park^{25/25}$ flies (discussed below, Figure 8B).

Elevated MTF-1 expression confers resistance to oxidative stress and restores metal homeostasis

Parkin mutant flies display high levels of ROS indicative of intrinsic oxidative stress (23, 72). In agreement with MTF-1 having an antioxidant function, we observed a substantial decrease of ROS levels in the heads of *parkin* mutants expressing an MTF-1 transgene: ROS dropped to approximately half of the levels in $park^{25/25}$ flies (Figure 8A). Previously we had observed that limiting the availability of redox-active metals, achieved by supplementing the food with chelators for copper and iron (BCS and BPS, respectively), also increased the lifespan of *parkin* mutant flies (55). Furthermore, the *w;tub-MTF1;*$park^{25/25}$ flies raised on the metal-chelator-supplemented food displayed a somewhat lower ROS level (Figure 8A). ROS levels were not significantly changed in heterozygous control flies, either upon ubiquitous MTF-1 overexpression or following dietary intake of metal-chelators. The *MTF-1* knockout flies showed the highest levels of ROS, probably due to the reduced expression of MTF-1-dependent antioxidant genes such as metallothioneins (Figure 8A). Metals like zinc and the redox active copper and iron are required in trace amounts for several structural and biological processes in organisms (66). $Park^{25/25}$ flies display not only reduced basal levels of zinc (see also (56)) but also of copper and iron in comparison to control flies (Figure 8B). Tubulin-driven MTF-1 expression in $park^{25/25}$ restores the basal level of these metals (Figure 8B). Concentrations of cadmium are generally low since it is a non-essential, toxic metal. Upon supplementing fly food with metals, their levels became quite similar in all three genotypes tested (Figure 8C). This is particularly important in the case of zinc supplementation, which we had previously shown to improve the condition of *parkin* mutant *Drosophila* (56).

Human MTF-1 or elevated metallothionein expression also improve condition of *parkin* mutants

Human MTF-1 has been expressed in *Drosophila* and shown to largely, but not completely, rescue the metal sensitivity of *Drosophila* lacking its endogenous MTF-1 (6). We therefore tested the effect of *actin-Gal4*-driven hMTF-1 expression in a *parkin* mutant background. Indeed the lifespan of *parkin* mutants was increased from

a median of 7 days to 19 days, which is close to the 21 days obtained with elevated *Drosophila*-MTF-1 expression (Figure 9). Other rescue effects paralleled those observed with elevated *Drosophila* MTF-1 expression (see above) but were less pronounced (data not shown).

Metallothionein genes are the major targets of MTF-1; in *Drosophila*, metallothionein A (*MtnA*) shows the strongest expression. Thus, we also tested if overexpression of *MtnA* in a *parkin* mutant background exerted a similar beneficial effect as *MTF-1* overexpression. To this end we crossed *inter se* three independent lines with tubulin-driven MtnA overexpression in a *parkin* heterozygous background to raise *parkin* homozygous knockouts. The median lifespan of the *parkin* mutants was extended up to 17 days (Figure 9) but other rescue effects associated with elevated MTF-1 expression (discussed in results) were not observed.

Discussion

Here we show that metal responsive transcription factor (MTF-1) plays a crucial role in modulating the severity of a Parkin loss-of-function phenotype. On the one hand, the combined loss of Parkin and MTF-1 is not viable, i.e., displays synthetic lethality. On the other hand, elevated expression of MTF-1 dramatically improves the condition of *parkin* mutant flies: there is an overall extension of life span, females regain normal fertility, and the motoric abilities of flies improve to the point that they can walk fast and even display short episodes of flight. The latter is noteworthy since flight muscles have a high energy consumption that depends on robust mitochondrial function. At the histological level, the degenerated mitochondria characteristically seen in *parkin* mutants are rescued to a more regular, electron-dense structure following MTF-1 overexpression. Additionally, flight muscles have an improved myofibrillar arrangement. In earlier studies, mitochondrial and muscle degeneration observed in *parkin* mutant flies were proposed to be a result of excessive oxidative stress (24, 48). Mitochondrial malfunction can indeed result in an increased susceptibility to oxygen radical damage, and mitochondria-associated increase in ROS production has been implicated in Parkinson's disease (19, 26, 73). In line with this concept, the major target genes of MTF-1 are metallothioneins (MTs), which encode small, cysteine-rich proteins that can scavenge heavy metals, notably the

redox-active copper, and ROS. The elevated basal level of *MTF-1* and MT transcripts in *parkin* mutant flies can thus be seen as a compensatory attempt to counteract enhanced ROS levels (24). In accordance with such a scenario is the rescue effect seen upon food supplementation with the antioxidant N-acetylcysteine, which however falls short of the dramatic effect seen with elevated MTF-1 expression. Besides metallothioneins and a number of other stress-associated genes, *ferritin* genes are a major target of MTF-1 in *Drosophila* (74). Ferritins are well-characterized iron binding proteins which keep iron in a soluble and non-toxic form in the cell. Thus ROS production by redox-active iron might be lowered via upregulation of *ferritin* levels. The multiple targets of MTF-1 help to explain why overexpression of metallothionein alone was less effective than MTF-1 overexpression in improving the condition of *parkin* mutants. In a separate series of experiments we found that strong expression of an ortholog, the human MTF-1, also increased lifespan, rescued female fertility and improved locomotion ability, although the effects were less pronounced than with *Drosophila's* own MTF-1, presumably due to the evolutionary distance between mammals and insects. This is in line with previous findings from our laboratory that mammalian and *Drosophila MTF-1* transgenes are able to largely, but not completely, compensate for each other's absence (6).

Although we observed remarkable improvements following elevated MTF-1 expression, a complete rescue of *parkin* mutants, including male fertility and sustained flight ability, was only observed with a *parkin* transgene. Furthermore, it has to be pointed out that although *MTF-1* knockout flies display high ROS levels, they show no signs of Parkinson's disease-like symptoms. Together these findings are consistent with the idea that oxidative stress is an important but not the sole culprit in PD etiology (1, 14, 49). Nevertheless, the dramatic effect of MTF-1 on a *parkin* loss-of-function mutation underscores the importance of this transcriptional regulator in cellular stress response.

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure Legends

Figure 1. Scheme of genetic cross to obtain strong MTF-1 expression in double mutant background. An MTF-1 cDNA transgene driven by the *tubulin* promoter was combined with homozygous null mutations of both *MTF-1* (*MTF-1*$^{-}$) (17) and *parkin* (*park*^{25}) (24); progeny in red. The *Sp* marker on the second chromosome gives a uniform-length side-bristle phenotype and the *CyO* second chromosome balancer results in curly wings. *TM3* and *TM6B* are third chromosome balancers which display a serrated wing phenotype and a dense side bristle phenotype, respectively. Balancers are lethal in homozygous form; progeny marked with a cross indicate lethality of that particular genotype. Chromosome 1 is wild type.

Figure 2. Enhanced lifespan of *parkin* mutants expressing an MTF-1 transgene. (A) A cDNA transgene of *Drosophila*-MTF-1 driven by the ubiquitously active *tubulin* enhancer/promoter prolongs the lifespan of *parkin* mutants (*park*$^{25/25}$) up to 41 days. For survival of *park*$^{25/25}$ vs. *tub-MTF-1, park*$^{25/25}$ flies, $p<0.001$. (B) Strong expression of a UAS-MTF-1 transgene driven by actin-Gal4 in a *parkin* mutant background also extends lifespan, as tested in multiple sets of *Act-Gal4; UAS-MTF-1*, *park*$^{25/25}$ flies. In both (A) and (B), heterozygous *park*$^{25/+}$ flies with MTF-1 overexpression had a similar lifespan as *park*$^{25/+}$ controls (data not shown).

Figure 3. Elevated MTF-1 expression restores ovary structures and restores

fertility of *parkin* mutant females. (A-A'') Normal ovariole structures of fertile control female flies ($park^{25/+}$). (B-B''') Infertile female *parkin* mutants ($park^{25/25}$) have a distorted ovary structure with very few mature eggs. (C-C'') Upon expression of a tubulin-driven *MTF-1* transgene ovary structures are normalised, resulting in normal fertility. a, anterior with germarium; p, posterior with vitellarium; broken arrows indicate mature eggs; full arrows developing stages of eggs. Phalloidin (red) stains tubulin structures and Toto (blue) stains the nuclei.

Figure 4. MTF-1 transgene expression restores the climbing ability of *parkin* mutants. Tubulin enhancer/promoter-driven MTF-1 expression largely rescues the locomotion ability of $park^{25/25}$ flies. In control flies, MTF-1 overexpression does not further improve climbing ability. Data shown represents mean value ± standard deviation of each group tested every other day up to 8 days. Asterisks show highly significant differences between *parkin* control ($park^{25/+}$) and *parkin* mutant ($park^{25/25}$) flies on each day of the assay ($p< 0.0001$).

Figure 5. Strong MTF-1 expression improves muscle and mitochondrial morphology of *parkin* mutants. (A-C) Transverse sections of indirect flight muscles (IFMs) show well preserved muscle in $park^{25/+}$ heterozygous controls with a regular myofibril arrangement (white arrows) and many electron-dense mitochondria (broken red arrows). (D-F) $park^{25/25}$ adult IFMs show an irregular myofibrillar arrangement with diffuse Z-lines and M-bands and numerous vacuoles. Mitochondria are swollen with fragmented cristae (red arrows). (G-I) Myofibril and mitochondrial integrity of *parkin* mutants is restored by MTF-1 overexpression. Mitochondria are more dense (broken white arrows) and muscle structure is more regular (broken green arrows), although occasional vacuoles are observed (green arrows). The scale bar is for top and middle panels while the bottom panel is at a higher magnification, shown for clarity.

Figure 6. MTF-1 activity is upregulated in a *parkin* deficient background. Top: Transgenic *MtnA/EYFP* reporter gene (6). Bottom: (A-B) *MtnA-EYFP,* $park^{25/+}$ and (C-D) *MtnA-EYFP,* $park^{25/25}$. Pictures of 1-2 day old adult flies were taken at 730 ms exposure with a Leica fluorescence microscope. 'a' and 'p' show anterior and posterior ends, respectively.

Figure 7. Increased *MTF-1* and metallothionein (*MtnB*) transcript levels in *parkin* mutants that also express an *MTF-1* transgene. Real-time transcript-levels of (A) *MTF-1*, (B) *MtnB*, (C) *parkin* and (D) *foi* in $park^{25/+}$ and $park^{25/25}$ and *w; tub-MTF-1,* $park^{25/25}$ and *Act-Gal4; UAS-MTF-1;* $park^{25/+}$ and *Act-Gal4; UAS-MTF-1;* $park^{25/25}$ flies with *MTF-1* knockout flies as an appropriate control. All flies were raised on normal food (NF). *MTF-1* null flies show no *MtnB* and *parkin* null flies show no *parkin* transcripts. (B) MTF-1 overexpression from the tubulin enhancer/promoter or via the UAS-Act-Gal4 system induced a 234-fold and 270-fold increase in MtnB transcripts, respectively. *** indicates significant difference between *parkin* mutant adult flies and *parkin* mutants with elevated MTF-1 expression ($p<0.0001$).

Figure 8. MTF-1 reduces reactive oxygen species (ROS) levels in *parkin* mutants and restores metal homeostasis. (A) $park^{25/25}$ flies (NF) show high amounts of ROS. MTF-1 transgene expression, or treatment with chelators of redox-active metals (BPS and BCS) reduce ROS levels. *parkin* heterozygous controls ($park^{25/+}$) do not show significant differences in ROS levels with or without MTF-1-overexpression or dietary supplementation with metal chelators. The *MTF-1* knockout shows the highest ROS level. ***, $p<0.001$ and **, $p<0.01$, chelator-supplemented compared to normal food for the same genotype (black stars), or compared to the same treatment between genotypes (red stars). (B) Elevated MTF-1 expression in a *parkin* mutant background restores normal basal levels of the essential trace metals copper, iron and zinc when flies are raised on NF (normal food). (C) Differences in metal content between controls, $park^{25/25}$ and *tub-MTF-1*, $park^{25/25}$ flies are largely leveled out by metal supplementation of the food.

Figure 9. Strong expression of human MTF-1 or of *Drosophila* metallothionein prolongs lifespan of *parkin* mutants. A human-MTF-1 transgene driven by *actin-Gal4* extends the median lifespan of *parkin* mutant flies from 7 (red) to 19 days (light blue). Direct overexpression of metallothionein MtnA by tubulin promoter also enhances the median lifespan of *parkin* mutants up to 17 days (green). An even better extension up to a median of 21 days is observed with a tubulin-driven trangene of *Drosophila* MTF-1 (dark blue). $park^{25/+}$ (black) and $park^{25/25}$ (red) serve as controls.

Table I. (A) Partial rescue of synthetic lethality of combined *parkin* and *MTF-1* mutants by N-acetylcysteine: *MTF-1, parkin* heterozygous parents were crossed *inter se* on food supplemented with 15mM NAC. **(B) Increased frequency of flies reaching adulthood among *parkin* mutants overexpressing MTF-1.** $park^{25/25}$ mutants were obtained from *parkin* heterozygous parents crossed *inter se*. In both (A) and (B), egg laying was allowed for two days by equal numbers of parents and eclosing progeny flies were counted thereafter. Three independent crosses were done.

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Figure 1

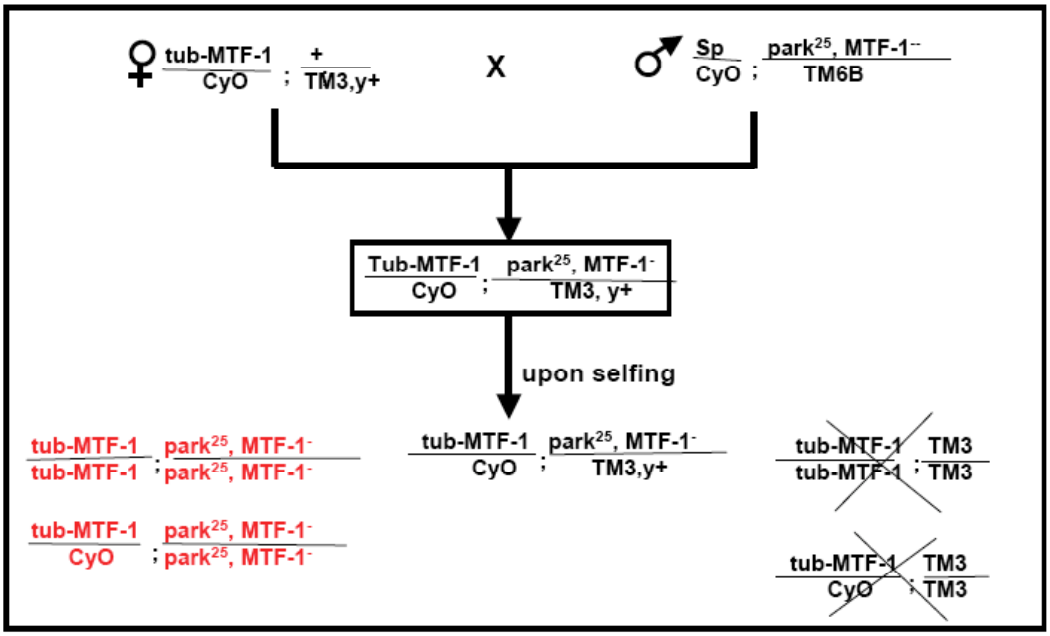
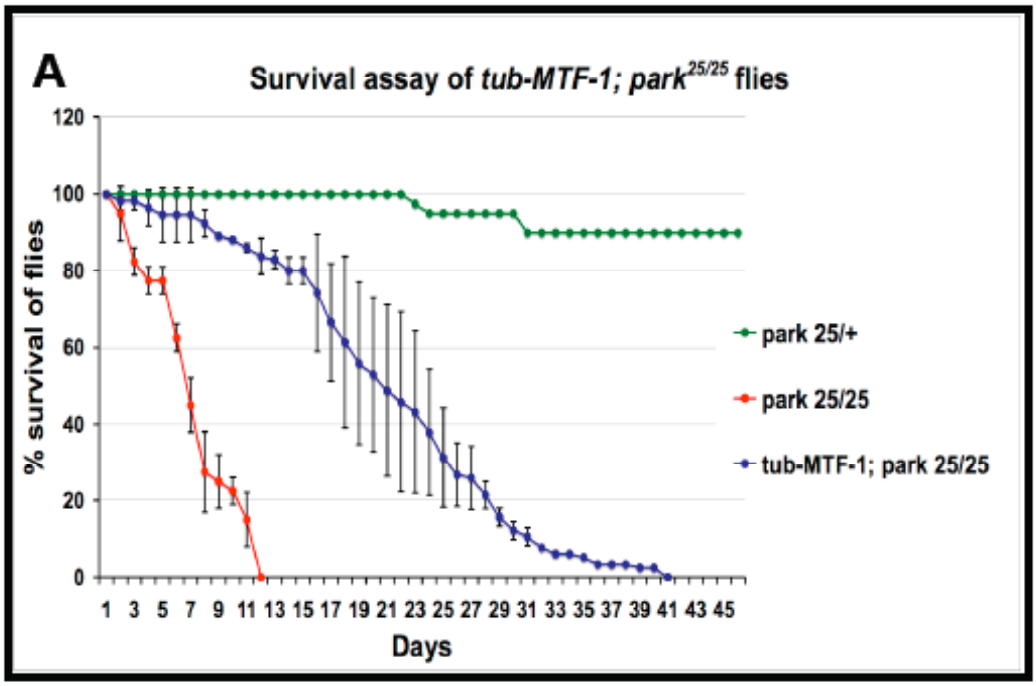

♀ tub-MTF-1 / CyO ; + / TM3,y+
X
♂ Sp / CyO ; park25, MTF-1-- / TM6B
Tub-MTF-1 / CyO ; park25, MTF-1- / TM3, y+
upon selfing
tub-MTF-1 / tub-MTF-1 ; park25, MTF-1- / park25, MTF-1-
tub-MTF-1 / CyO ; park25, MTF-1- / park25, MTF-1-
tub-MTF-1 / CyO ; park25, MTF-1- / TM3,y+
tub-MTF-1 / tub-MTF-1 ; TM3 / TM3
tub-MTF-1 / CyO ; TM3 / TM3

Figure 2


A
Survival assay of tub-MTF-1; park25/25 flies
% survival of flies
120
100
80
60
40
20
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45
Days
park 25/+
park 25/25
tub-MTF-1; park 25/25

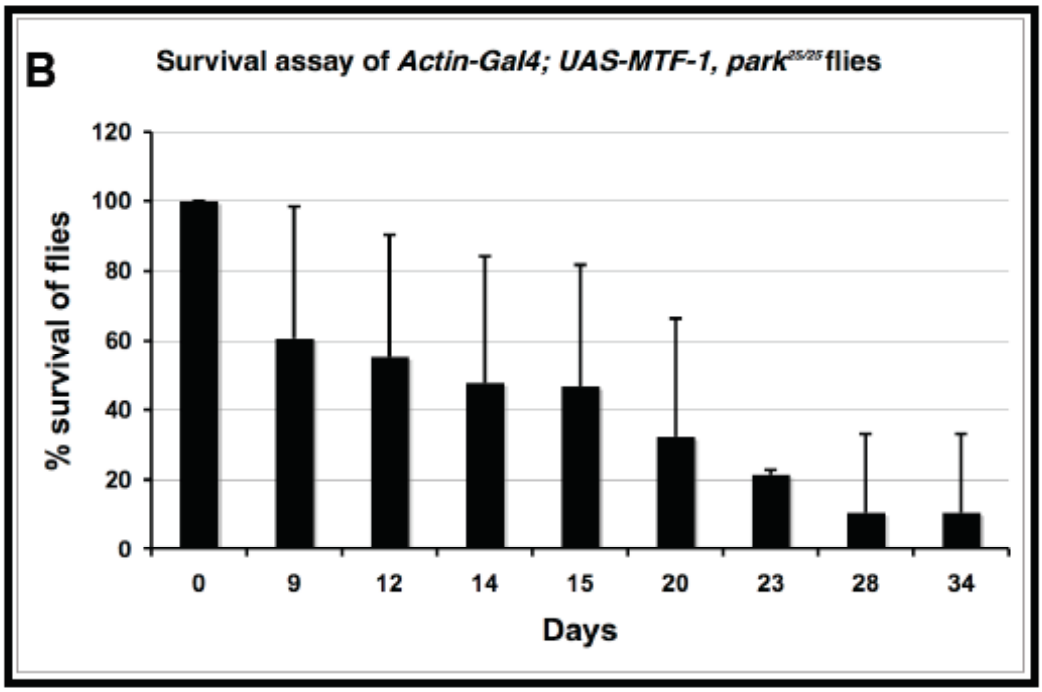

B
Survival assay of Actin-Gal4; UAS-MTF-1, park25/25 flies
% survival of flies
120
100
80
60
40
20
0
0
9
12
14
15
20
23
28
34
Days

Figure 3

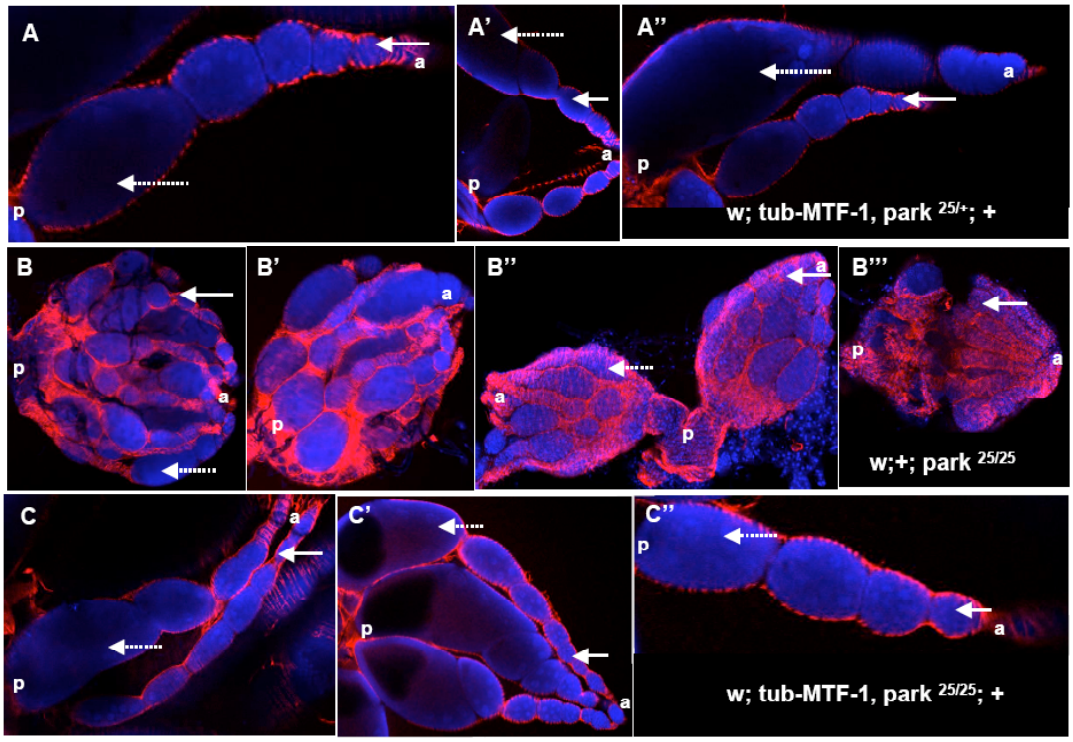

A
A'
A''
a
p
w; tub-MTF-1, park 25/+; +
B
B'
B''
B'''
w;+; park 25/25
C
C'
C''
w; tub-MTF-1, park 25/25; +

Figure 4

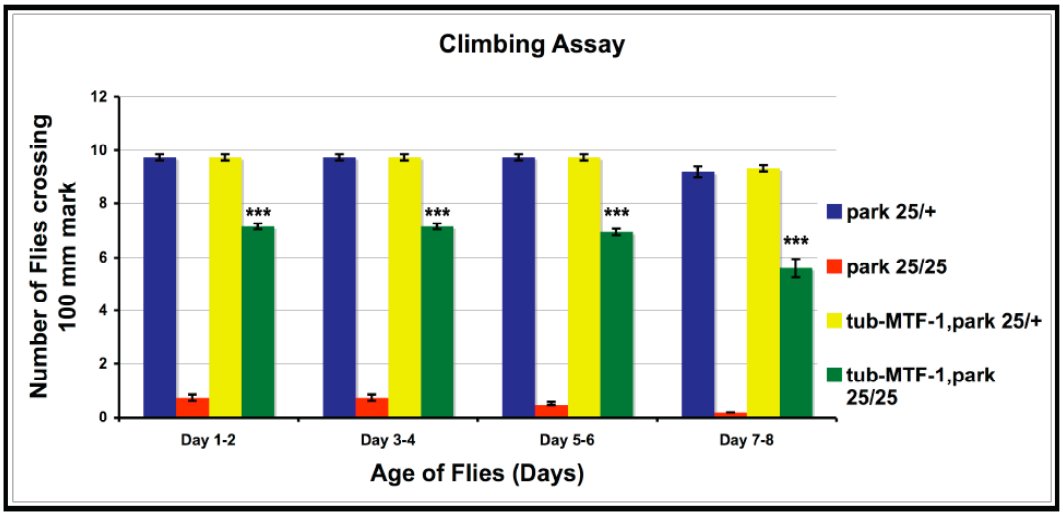

Climbing Assay
Number of Flies crossing 100 mm mark
12
10
8
6
4
2
0
***
***
***
***
Day 1-2
Day 3-4
Day 5-6
Day 7-8
Age of Flies (Days)
park 25/+
park 25/25
tub-MTF-1,park 25/+
tub-MTF-1,park 25/25

Figure 5

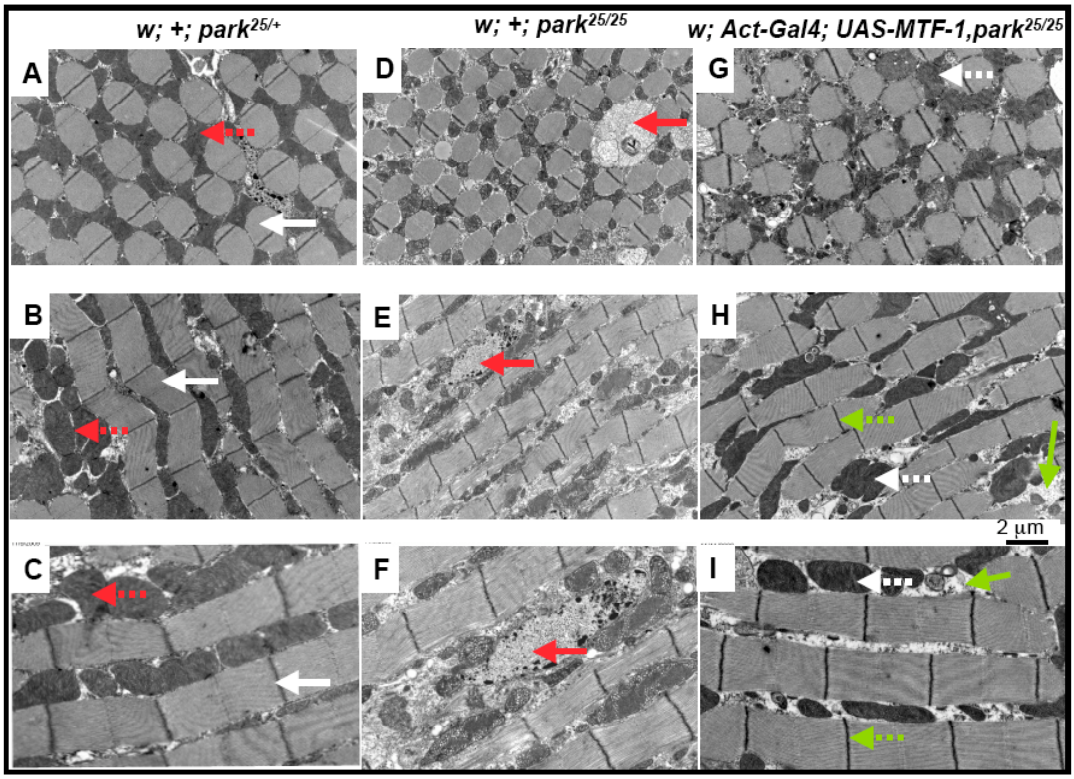

w; +; park25/+
w; +; park25/25
w; Act-Gal4; UAS-MTF-1,park25/25
A
B
C
D
E
F
G
H
I
2 µm

Figure 6

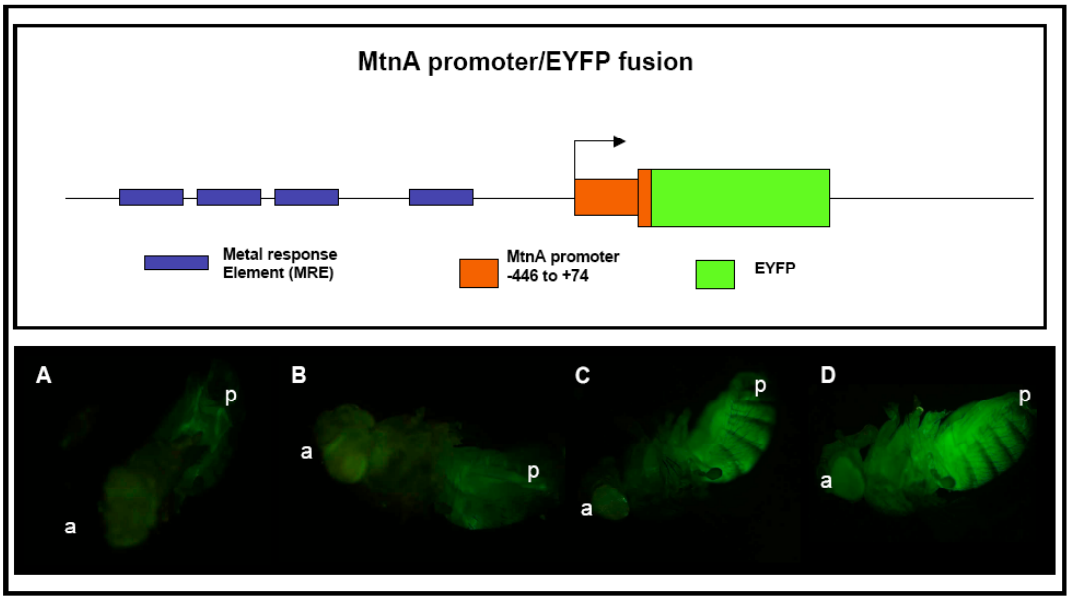

MtnA promoter/EYFP fusion
Metal response Element (MRE)
MtnA promoter -446 to +74
EYFP
A
p
a
B
a
p
C
p
a
D
p
a

Figure 7

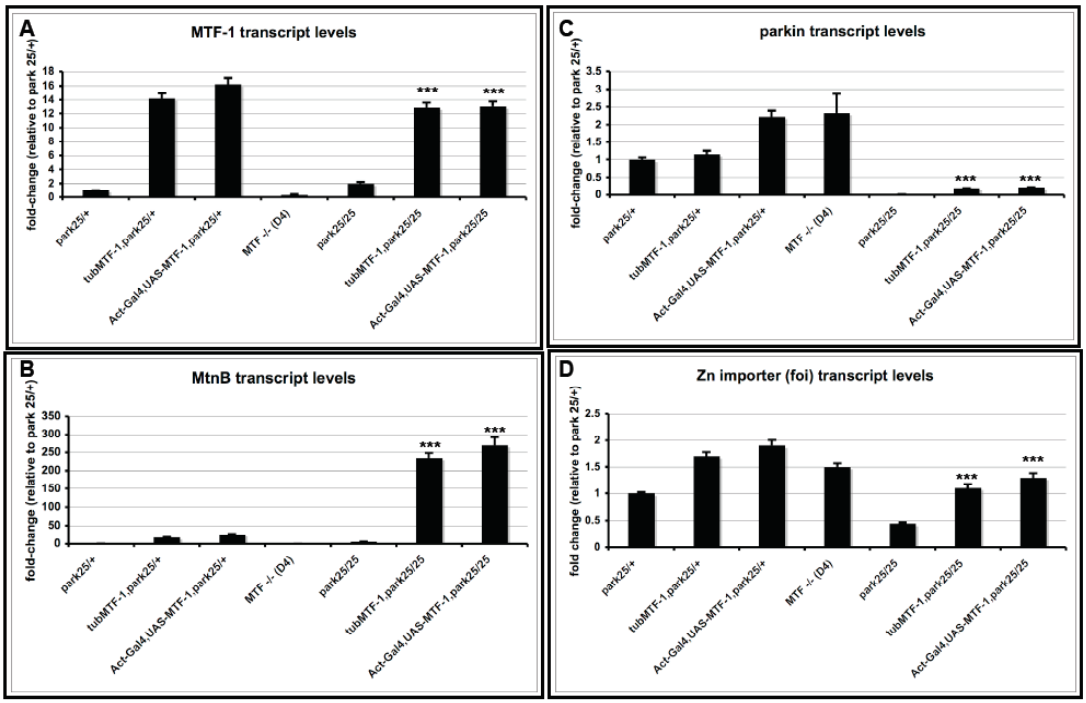

A
MTF-1 transcript levels
fold-change (relative to park 25/+)
park25/+
tubMTF-1,park25/+
Act-Gal4,UAS-MTF-1,park25/+
MTF -/- (D4)
park25/25
tubMTF-1,park25/25
Act-Gal4,UAS-MTF-1,park25/25
***
C
parkin transcript levels
B
MtnB transcript levels
D
Zn importer (foi) transcript levels
fold change (relative to park 25/+)

Figure 8

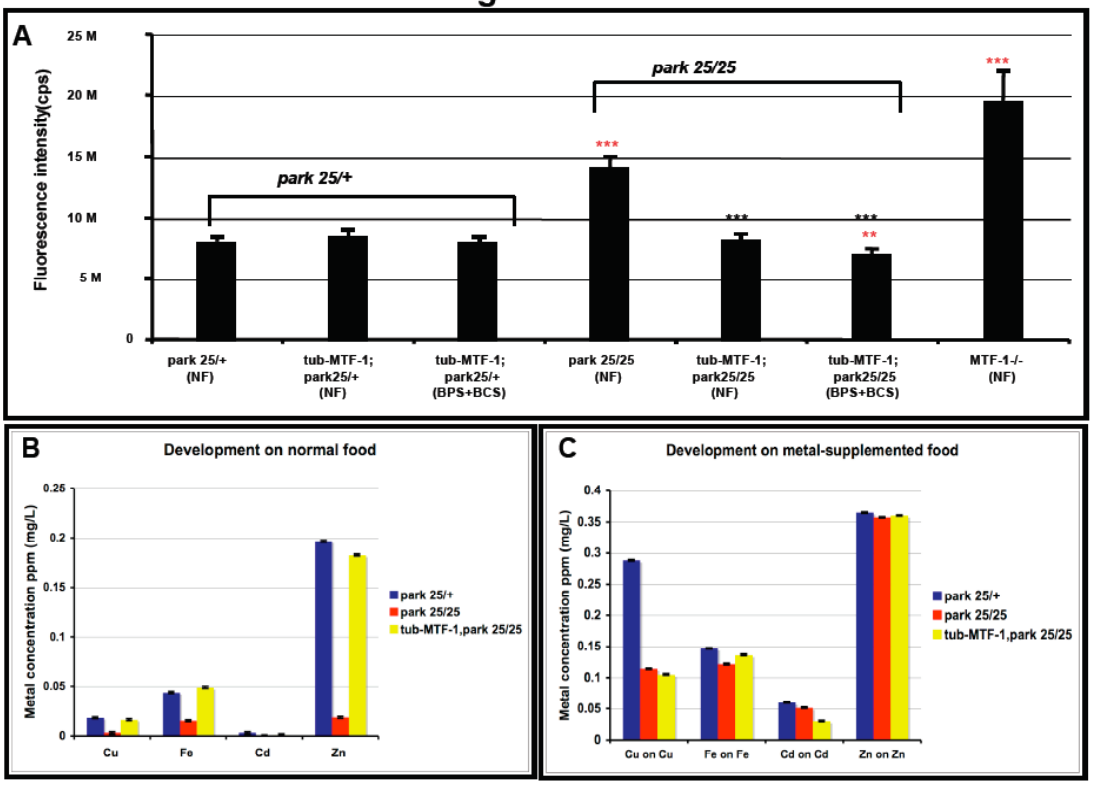

A
Fluorescence intensity(cps)
25 M
20 M
15 M
10 M
5 M
0
park 25/+
park 25/25
***
**
park 25/+ (NF)
tub-MTF-1; park25/+ (NF)
tub-MTF-1; park25/+ (BPS+BCS)
park 25/25 (NF)
tub-MTF-1; park25/25 (NF)
tub-MTF-1; park25/25 (BPS+BCS)
MTF-1-/- (NF)
B
Development on normal food
Metal concentration ppm (mg/L)
0.25
0.2
0.15
0.1
0.05
0
Cu
Fe
Cd
Zn
park 25/+
park 25/25
tub-MTF-1,park 25/25
C
Development on metal-supplemented food
Metal concentration ppm (mg/L)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Cu on Cu
Fe on Fe
Cd on Cd
Zn on Zn
park 25/+
park 25/25
tub-MTF-1,park 25/25

Figure 9

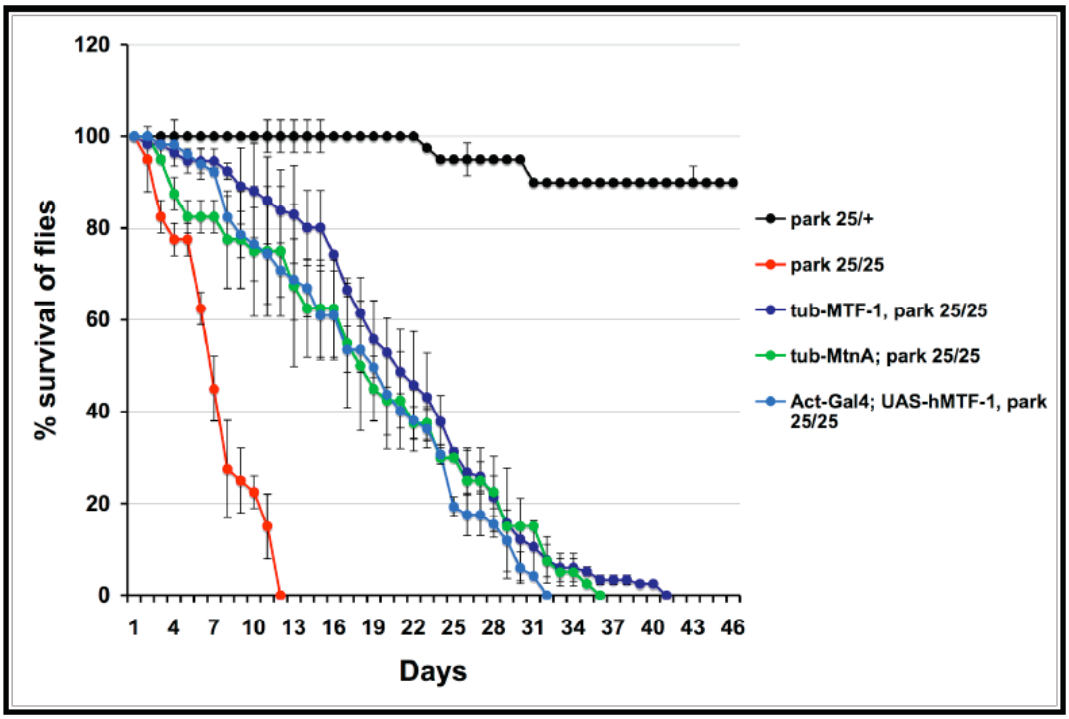

120
100
80
60
40
20
0
% survival of flies
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46
Days
park 25/+
park 25/25
tub-MTF-1, park 25/25
tub-MtnA; park 25/25
Act-Gal4; UAS-hMTF-1, park 25/25

Table 1A

15 mM NAC supplemented food	♀ *w; Sp/CyO; MTF-1^{-}, park^{25}/ TM3* X ♂ *w; Sp/CyO; MTF-1^{-}, park^{25}/ TM3*			
	Parkin and *MTF-1* heterozygous progeny: *w; Sp/CyO; MTF-1^{-}, park^{25}/ TM3*	*Parkin* and *MTF-1* homozygous progeny: *w; Sp/CyO; MTF-1$^{-/-}$,park$^{25/25}$*	Total number of progeny	Percentage of *parkin and MTF-1* homozygous progeny
Cross 1	62	14	76	18.4
Cross 2	79	19	98	19.4
Cross 3	68	16	84	19.0
Total	209	49	258	19.0

Table 1B

normal food	♀ *w; +; tub-MTF-1, park $^{25/+}$* X ♂ *w; +; tub-MTF-1, park $^{25/+}$*			
	Parkin and *MTF-1* heterozygous progeny: *w; +; tub-MTF-1, park $^{25/+}$*	*Parkin* and *MTF-1* homozygous progeny: *w; +; tub-MTF-1, park$^{25/25}$*	Total number of progeny	Percentage of *parkin and MTF-1* homozygous progeny
Cross 1	310	53	363	14.6
Cross 2	741	141	882	16.0
Cross 3	229	37	266	13.9
Total	1280	231	1511	15.3

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Zinc supplement greatly improves the condition of parkin mutant Drosophila.

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder in which oxidative stress is implicated as a major causative factor. Mutations in the gene encoding Parkin, a ubiquitin ligase, are responsible for a familial form of PD. In a Drosophila disease model lacking Parkin (park(25) null mutant), we tested the effect of zinc supplementation. Zinc is an essential trace metal and a component of many enzymes and transcriptional regulators. Unlike copper and iron, zinc is not redox-active and under most conditions serves as an antioxidant. We find that the condition of parkin mutants raised on zinc-supplemented food is greatly improved. At zinc concentrations where controls begin to show adverse effects as a result of the metal supplement, parkin mutants perform best, as manifested in a higher frequency of reaching adulthood, extended lifespan and improved motoric abilities.

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Short Communication

Zinc supplement greatly improves the condition of *parkin* mutant *Drosophila*

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder in which oxidative stress is implicated as a major causative factor. Mutations in the gene encoding Parkin, a ubiquitin ligase, are responsible for a familial form of PD. In a *Drosophila* disease model lacking Parkin ($park^{25}$ null mutant), we tested the effect of zinc supplementation. Zinc is an essential trace metal and a component of many enzymes and transcriptional regulators. Unlike copper and iron, zinc is not redox-active and under most conditions serves as an antioxidant. We find that the condition of *parkin* mutants raised on zinc-supplemented food is greatly improved. At zinc concentrations where controls begin to show adverse effects as a result of the metal supplement, *parkin* mutants perform best, as manifested in a higher frequency of reaching adulthood, extended lifespan and improved motoric abilities.

Keywords: antioxidant; metal homeostasis; metallothioneins; MTF-1; Parkinson's disease; zinc transporters.

Parkinson's disease (PD), characterized by the loss of dopaminergic (DA) neurons in the substantia nigra, is a progressive neurodegenerative disorder with the second highest incidence rate and is the most common age-related movement disorder (Olanow and Tatton, 1999; Dawson and Dawson, 2003; Greene et al., 2003). Both genetic and environmental factors contribute to its pathogenesis. Oxidative stress is considered to be a major factor in the pathogenesis of PD, as evidenced by an elevated content of redox-active iron and lipid peroxides in the diseased brain, impaired mitochondrial function, and alterations in the antioxidant defense mechanisms (Dexter et al., 1989; Jenner and Olanow, 1996; Greene et al., 2003; Pesah et al., 2004). Mutations in six genes, including *parkin* which encodes an E3 ubiquitin ligase, have been associated with rare, early-onset, familial forms of PD (West and Maidment, 2004; Gasser, 2005; Sang et al., 2007). Interestingly, some alleles of these genes might be susceptibility factors for environmental toxins (Choi et al., 2000; Warner and Schapira, 2003; Bueler, 2009).

In our study, we used a *Drosophila melanogaster* line in which the ortholog of the human *parkin* gene is disrupted by transposition of a P-element (Greene et al., 2003; Pesah et al., 2004). Parkin mutant flies present with male and female sterility (Riparbelli and Callaini, 2007), mitochondrial and muscle abnormalities, locomotor defects, an inability to fly owing to degeneration of indirect flight muscles, increased sensitivity to multiple stresses, including oxidative stress, and a severely reduced lifespan (Palacino et al., 2004; Greene et al., 2005; Whitworth et al., 2005). Some of these defects arise because *parkin* mutants have dysfunctional mitochondria with disturbances in the electron transport chain. In mice, in contrast to its pivotal role in humans (Choi et al., 2000), Parkin function does not seem to be critical for the survival of DA neurons (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004). Similarly, *Drosophila* that are null mutants for *parkin* do not generally display DA neuron loss (Greene et al., 2003; Pesah et al., 2004), although a partial loss of DA neurons in the PPL1 cluster of the brain has been reported (Whitworth et al., 2005).

In the late 1980s, the antioxidant function of the redox-inert metal zinc was recognized and proposed to be mediated by the protection of protein sulfhydryl groups and/or by competing against redox-active metals (Bray and Bettger, 1990). Additionally, zinc ions can upregulate the expression of metallothioneins, which owing to their high cysteine content can serve as antioxidants. Zinc has an established antiapoptotic function that minimizes ROS-induced cellular oxidative damage (Suzuki et al., 1991). This also occurs in the central nervous system, particularly in the brain (Kocaturk et al., 1996).

More than 70 different enzymes involved in the metabolism of biomolecules require zinc as a cofactor (Parkin, 2004). Zinc is an integral part of the hundreds of transcription factors that contain zinc finger domains (Berg and Shi, 1996), and it plays a role in cellular signal transduction and in modulation of synaptic neurotransmission. Zinc is critical for the growth and regulation of cells and alterations in zinc metabolism have been implicated in causing neurological dysfunctions on the one hand, and on the other hand providing neuroprotection. Maintenance of intracellular zinc homeostasis is thus an essential requirement in all living organisms (Valko et al., 2005).

The best characterized zinc-activated transcription factor is the metal response element-binding transcription factor-1,

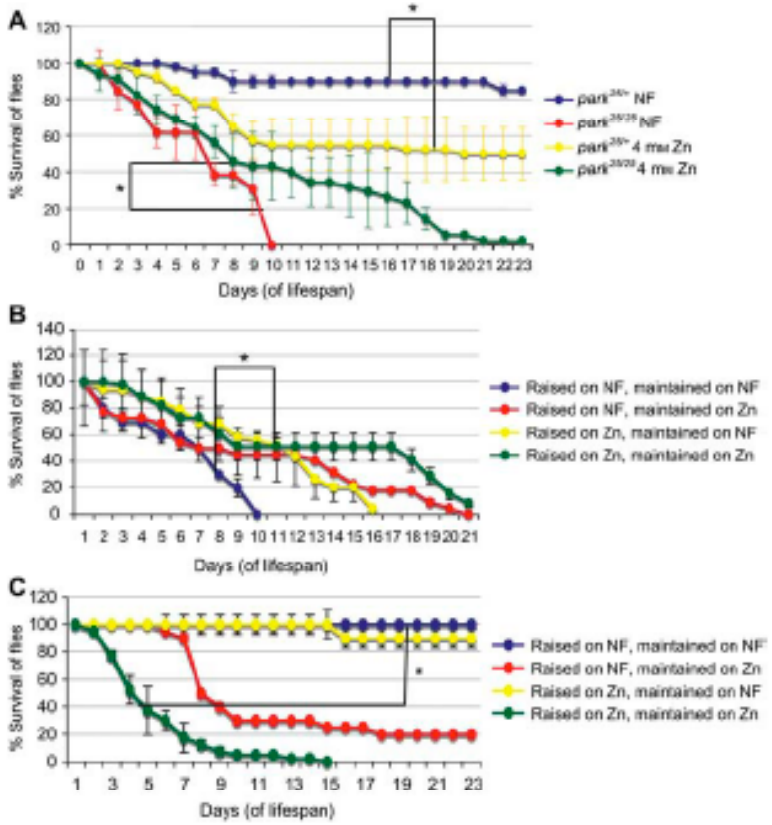

Figure 1 *Drosophila parkin* mutants show an enhanced lifespan on zinc-supplemented food.
(A) Zinc supplementation (4 mM $ZnCl_2$) increased the longevity of the *parkin* mutant flies ($park^{25/25}$). The maximum lifespan of 11 days on normal food (NF) increased to 23 days. Control flies ($park^{25/+}$) showed an opposite effect with a reduction of lifespan on zinc food. (B) Survival of $park^{25/25}$ flies and (C) of $park^{25/+}$ control flies raised and maintained on NF or zinc-supplemented food. Error bars indicate standard deviation. The significance between survival curves was analyzed using the Kaplan-Meier log-rank statistical test (*$p<0.01$).
Methods: for survival assays, NF was supplemented with zinc chloride to a final concentration of 4 mM. One- to two-day-old flies (20 per vial) were maintained at 25°C on a 12:12 h light/dark cycle for each genotype in triplicate vials. Surviving flies were transferred to fresh food vials every 2 days and counted daily. As a control, flies of the same genotype were grown on food without metal supplement. In each lifespan assay testing different conditions, the controls of $park^{25/+}$ and $park^{25/25}$ flies raised on NF were the same. The variations in the median lifespan of control flies in different experiments can be attributed to subtle experimental variations.

also referred to as metal-responsive transcription factor-1 (MTF-1). In response to zinc, MTF-1 translocates from the cytoplasm to the nucleus where it regulates the expression of several genes, notably metallothioneins and some zinc transporters (Andrews, 2000; Smirnova et al., 2000; Lichtlen and Schaffner, 2001; Saydam et al., 2001). MTF-1 requires elevated zinc concentration for DNA binding. This property was exploited for the activation of MTF-1-dependent pro-

Table 1 Zinc increases the frequency of *parkin* mutant *Drosophila* reaching adulthood.

Food condition	Genotype	$park^{25/25}$/total progeny	% of $park^{25/25}$
Normal food	Set 1 $park^{25/25}$	31/1116	2.8%
	Set 2 $park^{25/25}$	10/450	2.2%
4 mM Zn	Set 1 $park^{25/25}$	110/611	18%
	Set 2 $park^{25/25}$	186/956	19%

For the analysis of eclosure frequency, egg laying was allowed for 6 days and the parent population (10 males and 10 females each of $park^{25/+}$) was the same in all vials of normal food (NF) or zinc (Zn), and progeny flies were counted at the same time.

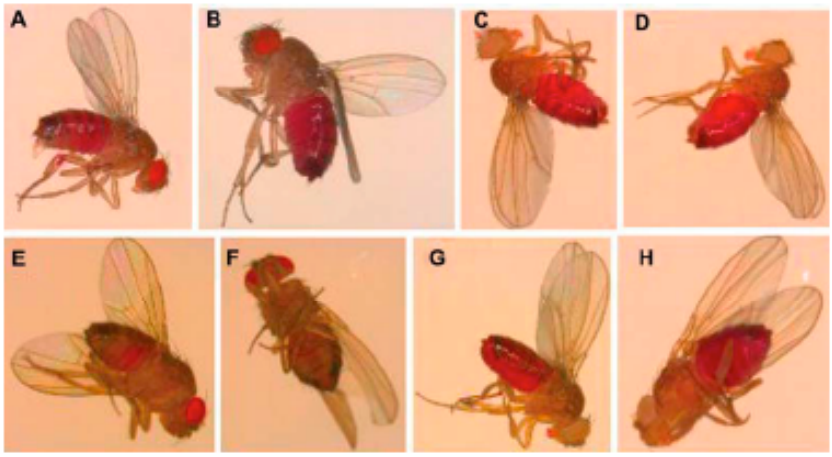

Figure 2 Gustatory assay with adult flies on normal or zinc-supplemented food with acid red dye as a marker.
(A, B) $park^{25/+}$ control flies and (C, D) $park^{25/25}$ flies show the same feeding behavior on normal food, exemplified by the fully red abdomens. A food supplement of 4 mM Zn (E, F) altered feeding behavior of $park^{25/+}$ (only partially red abdomens) but no change in the behavior of $park^{25/25}$ flies was observed (G, H) (fully red abdomens). The mutants are recognized by white eyes because of the loss of w^+ (red eyes) which are present in control flies.
Methods: the adult gustatory assay was essentially carried out as described by A. Hilliker and colleagues (Bahadorani, 2008). Briefly, newly eclosed flies were reared on normal food for 2–3 days, then starved for 18 h on Whatman paper soaked with distilled water. After this treatment, starved flies (20 per vial) were transferred onto zinc-supplemented food with 0.2% sulforhodamine B sodium salt (acid red) for 2 h. For control flies, culture medium was supplemented with 0.2% acid red, without metal supplement. After 2 h of feeding at optimum temperature (25°C) and relative humidity, flies were anesthetized and the degree of abdomen redness was visually inspected. Abdomen redness was used as an indicator of the amount of food taken up.

moters by zinc and other metals in a cell-free transcription system (Zhang et al., 2003).

Zinc is not redox-active, but nevertheless toxic when in excess (Beyersmann and Haase, 2001). Acute zinc toxicity is rare but has been reported (Duncan et al., 1992; Whittaker, 1998; Prasad et al., 1999). If the extracellular concentration of zinc exceeds the capacity of zinc homeostasis mechanisms, it becomes cytotoxic and an excess of free intracellular zinc can trigger apoptosis (Choi et al., 1988; Duncan et al., 1992; Kim et al., 1999; Beyersmann and Haase, 2001; Wilhelm et al., 2001; Beyersmann, 2002; Walther et al., 2003). Zinc transport in *Drosophila*, as in vertebrates, is mediated by two families of solute linked carrier proteins: zinc importers (ZIPs), which function in the uptake of zinc to the cytoplasm, and zinc exporters (ZnTs), which reduce cytoplasmic zinc concentrations by promoting zinc efflux (Liuzzi and Cousins, 2004; Yepiskoposyan et al., 2006). More than 10 zinc transporter genes are annotated in *Drosophila melanogaster* based on their sequence similarities to vertebrate zinc transporters. The ZIP family gene *foi* (*fear of intimacy*) was characterized in *Drosophila* and shown to be a zinc importer that is critical during development (Moore et al., 1998; Mathews et al., 2005). Transcriptional responses to zinc in *Drosophila* larvae were analyzed in our laboratory (Yepiskoposyan et al., 2006). Apart from the expected upregulation of metallothioneins and the zinc exporters *ZnT35C* and *ZnT63C*, there was also an induction of neurotransmitters, detoxification enzymes (such as glutathione S-transferase), ferritin and chaperone encoding genes.

We found that whereas *parkin* mutant flies readily feed on high-zinc food, their wild type counterparts avoid zinc-loaded food. The mutants also had an increased survival rate on zinc-supplemented food, which prompted us to investigate their response to zinc in more detail.

With standard 'normal food' (NF), *parkin* mutant flies have a median lifespan of 6 days with a maximum of 11 days (Figure 1A). Zinc supplementation in the form of zinc chloride increased the lifespan of *parkin* mutants. When maintained on supplements of 4 mM Zn, the mutant flies survived up to 23 days with a median lifespan of 8 days (Figure 1). This increase was as a result of zinc and not chloride ions, as similar survival assays on NaCl-supplemented medium did not extend lifespan (data not shown). Zinc supplementation also increases the eclosion frequency of *parkin* mutants, from 2.5% to 19%, i.e., close to 25% which is the expected frequency of *parkin* mutants in the progeny of *parkin* heterozygous parents (Table 1). In contrast, heterozygous control flies did not draw any benefit from zinc-supplemented food: if kept on food with 4 mM $ZnCl_2$, the median lifespan reduced significantly to 17 days, whereas on NF, 80% were still alive at the end of the experiment (23 days) (Figure 1A). Similar adverse effects of zinc load were

observed with wild type *yw* and *OregonR* flies (data not shown). To determine if zinc has a stronger effect during development from eggs to adults or during the adult feeding stage, we followed the lifespan of both *parkin* mutant and *parkin* heterozygous flies under four conditions: (i) development and adult maintenance in zinc supplement, (ii) raising the flies on NF until adult stage and then maintaining them on zinc-supplemented food post-eclosion, (iii) raising the flies on zinc food but maintaining them on NF after eclosion, and (iv) raising and maintaining the flies on NF. We observed that the strongest positive effect of zinc on $park^{25/25}$ flies was when they were both raised and maintained on 4 mM Zn-supplemented food (Figure 1B). The strongest negative effect of zinc on $park^{25/+}$ control flies was also observed under these conditions (Figure 1C). Development of $park^{25/+}$ and *OregonR* wild type flies was delayed but generally less affected by zinc than the survival of adults; egg transfer (200 each) from NF to vials containing increasing concentrations of zinc resulted in an equal percentage of eclosing adults in NF, 4 and 6 mM Zn but none developed in 10 mM Zn (data not shown; see also Egli et al., 2003).

parkin mutants might sense a deficiency in and/or a rescuing effect of zinc, and in response to this eat normal amounts of zinc-supplemented food unlike heterozygous or wild type flies. This was indicated by a visual inspection of adult *Drosophila* in a gustatory test and was quantitatively confirmed by the measurement of total zinc uptake by flies (Figures 2 and 3). On NF, the zinc content in heads of *parkin* mutant flies was much lower than in controls, consistent with a zinc deficiency in the mutants. Zinc supplementation indeed resulted in an, albeit minor, increase of zinc content in mutant heads (Figure 3A), whereas in thoraces the zinc content was similar in mutant and control flies (Figure 3B). Interestingly, the abdomens of $park^{25/25}$ flies fed on zinc food showed a 10-fold increase in zinc levels compared with flies fed on NF (Figure 3C). Such an effect was, however, not observed in the control flies which only showed a minor increase in zinc levels upon zinc feeding, which is in line with their tendency to avoid zinc-loaded food.

We also determined the transcript levels of *parkin*, MTF-1 and some other genes involved in zinc import/export (Figure 4). As expected, *parkin* mutants have no detectable *parkin* transcripts. In comparison to control flies, transcript levels of the metallothionein *MtnB* are higher in *parkin* mutants (Figure 4A,B). Zinc supplementation induced *MtnB* transcripts 24-fold in *parkin* mutants; in control flies the increase was 14-fold (Figure 4B). Boosting expression of metallothioneins, which act as ROS scavengers, is one way zinc could play an antioxidant role. The elevated metallothionein levels could also explain at least in part why an increased zinc concentration is not toxic to the *parkin* mutants. We also determined the transcript levels of three zinc transporters, the exporters *ZnT35C* and *ZnT63C* and the importer *foi* (Mathews et al., 2005; Yepiskoposyan et al., 2006) both in *parkin* mutants and in the heterozygous controls. The most conspicuous difference was observed with the exporter gene *ZnT35C*, a known target of MTF-1. In *parkin* mutants, expression was dramatically reduced compared to controls and not responsive to zinc. In heterozygous controls, zinc supplement resulted in a 1.5-fold upregulation, which indicates a response to zinc overload (Figure 4C). An increased expression of zinc exporters in control flies is in

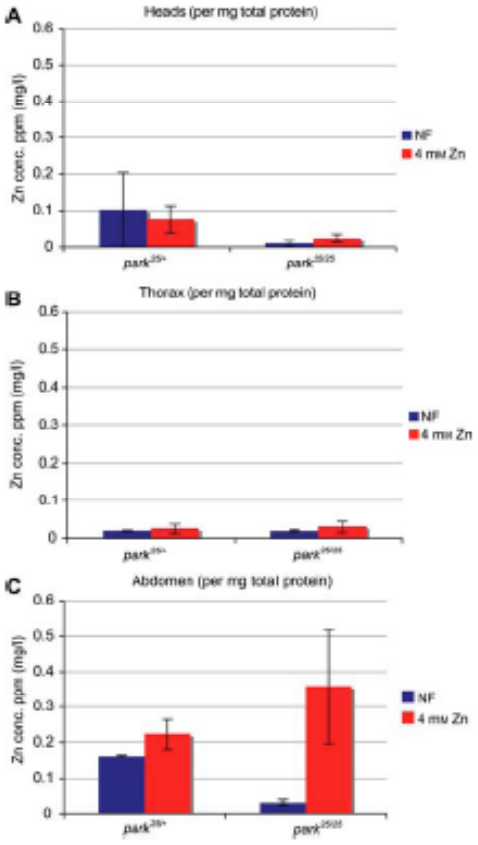

Figure 3 Quantification of Zn content in the different body parts of $park^{25/+}$ control flies and $park^{25/25}$ flies.

Flies were raised on normal food (NF) or zinc-supplemented food (4 mM Zn). (A) Heads, (B) thoraces, (C) abdomens.

Methods: female flies were allowed to lay eggs on zinc-supplemented (4 mM $ZnCl_2$) fly food for 6 days and removed afterwards. The resulting progeny was collected at regular intervals and the percentage of eclosing *parkin* homozygous mutants and heterozygous controls were calculated each time. These progeny were then frozen and stored until the required number of 200 flies was obtained in triplicate for each genotype. Frozen flies were then dissected to separate their heads, thoraces and abdomens which were collected separately. Each sample set was then subjected to homogenization using cold protein homogenization buffer (0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, 0.5 mM PMSF protease inhibitor at pH 7.4) and the samples were normalized for protein content. A highly sensitivity flame atomic absorption spectrophotometer (FAAS; GTA-120/PSD-120, Varian Australia Pty Ltd, Mulgrave, VIC, Australia) was used to detect the zinc content in each body part of each genotype assessed.

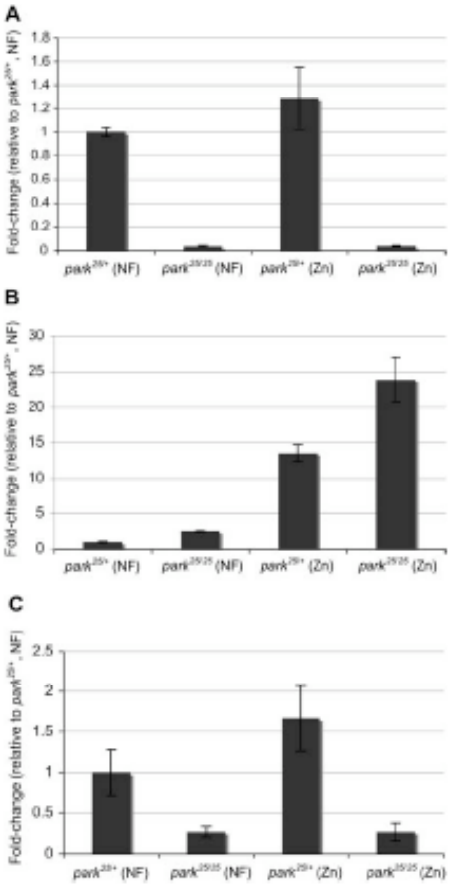

Figure 4 Real-time analysis of *parkin* transcript levels.
(A) In $park^{25/25}$ flies no *parkin* transcripts are detectable. In $park^{25/+}$ *parkin* transcripts are, if anything, elevated in flies raised on zinc food. (B) $park^{25/25}$ flies have increased levels of metallothionein *MtnB* transcripts in NF compared to control flies. Zinc induces *MtnB* in both genotypes, but to a higher level in $park^{25/25}$. (C) The zinc exporter, *ZnT35C*, shows increased transcript levels in $park^{25/+}$ on zinc food, which is not observed in $park^{25/25}$ flies.
Methods: total RNA was purified from the adult *Drosophila* tissue using the nucleospin RNA II protocol and eluted in 60 µl RNase-free water. cDNA was prepared using the transcriptor high fidelity cDNA synthesis kit from Roche (Mannheim, Germany). The cDNA obtained was further purified using the AM 1906 DNA-free kit (Ambion, Rotkreuz, Switzerland). This was then used for analysis by real-time PCR on the Tecan Genesis 200/8 robot (Tecan, Männedorf, Switzerland) using the Mesa Green qPCR Mastermix Plus (Eurogentec, Seraing, Belgium) for SYBR assays. The qPCR run was performed on an Applied Biosystems machine (ABI Prism SDS 7900 HAT; Seraing, Belgium) in a 384-well format with a reaction volume of 10 µl. All fold-change values are normalized to corresponding $park^{25/+}$ values on normal food (NF). Housekeeping genes used for reference were *actin5C*, *sec24* and *GAPDH*. The primer sequences used for the transcripts quantified were: for *parkin*, 5′-AAG ATC ATA TTT GCC GGT AAG GAA-3′ and 5′-CGC TTT GCT GAC CCA AGT C-3′ amplify a 73-bp fragment only from the *parkin* heterozygous control flies; for *MtnB*, 5′-TTG CAA GGG TTG TGG AAC AAA-3′ and 5′-TGC AGG CGC AGT TGT CC-3′ amplify a 65-bp fragment, and for *ZnT35C*, 5′-GCT CTC GCC GAT CTG AAA GT-3′ and 5′-CGC ACC GAC AAG TGT TTC TTA TA-3′ amplify a 75-bp fragment.

agreement with their avoidance of zinc-supplemented food. Transcripts of *ZnT63C* and, somewhat unexpectedly of *foi*, were also lower in *parkin* mutants compared to *parkin* heterozygous controls (data not shown). Taken together, these data suggest that zinc homeostasis is distorted in *parkin* mutants, with the effect that zinc supplement in food produces a strong phenotypic rescue effect. It remains to be seen whether zinc supplementation also has a positive effect in a mouse model of familial PD and ultimately in Parkinson's patients.

Acknowledgments

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Conclusion and outlook

This work presents several new insights into the interplay between *parkin* and MTF-1 gene products and the beneficial effect of increased zinc in *parkin* mutant flies. The three major advancements outlined here are: (i) the extension of lifespan of *parkin* mutant flies by chelators of redox-active metals; (ii) characterization of the dramatic rescue effect of MTF-1 in the *parkin* deficient flies and (iii) the beneficial effect of zinc in ameliorating the effect of a *parkin* null mutation in *Drosophila*.

Since it was previously known that *parkin* mutant *Drosophila* suffer from high oxidative stress, we tested the effect of chelating redox-active metals (copper/iron) from the flies. Interestingly, as discussed before, we observed a dramatic rescue of the lifespan of the sick flies which demonstrated reduced ROS levels. Contrary to our expectations, *parkin* mutant flies fed with food containing high micromolar concentrations of redox-active metals like iron or copper did not display any obvious detrimental effects beyond their severe condition already observed with normal food.

To determine the exact reason why the *parkin* null *Drosophila* benefit from metal chelation we proceeded to investigate the effects of modulating the dosage of MTF-1 which is required for restoring homeostasis in a physiologically disturbed cellular system. Further, while testing the effect of the essential trace element, zinc on *parkin* mutants, we found the unexpected but nevertheless consistent result that zinc supplement ameliorated the *parkin* mutant phenotype, whereby the *parkin*

mutants seem to sense that high zinc levels help improve the quality of their life.

Upon MTF-1 overexpression, we were able to rescue, largely or completely, a number of phenotypic traits of a *Drosophila* model of Parkinson's disease, namely; locomotion disability associated with distorted muscle/mitochondrial morphology; significantly reduced lifespan; female infertility, and low eclosing frequency of *parkin* mutant adults. The metal responsive transcription factor MTF-1 plays a pivotal role in modulating the *parkin* phenotype: flies lacking both MTF-1 and *parkin* are not viable ("synthetic lethality"), while overexpression of MTF-1 has strong beneficial effects.

Zinc deprivation by chelation or starvation is known to cause cells to die, in many cell types by programmed cell death (Truong-Tran et al., 2001). Our results also indicate an imbalance of zinc homeostasis in the *parkin* mutant flies which can be counteracted by zinc supplement. By providing food with a high zinc concentration to the *parkin* mutant flies, we were able to rescue a few major phenotypic traits to a significant extent. These included an increase in the eclosing frequency of *parkin* mutant adults, a reduced developmental delay and an increased lifespan of mutant adults. However, a high zinc concentration in the food, while extending the lifespan of *parkin* mutants, was harmful for the *parkin* heterozygous control flies, as evidenced by a developmental delay and a reduced lifespan as well as a decreased propensity of flies to egg-laying. This seeming zinc paradox prompted us to investigate the behavioural response of both *parkin* mutants and control flies towards higher zinc levels. If the extracellular concentration of zinc exceeds the capacity of cellular zinc homeostasis, it becomes cytotoxic and the subsequent

increased concentration of intracellular zinc triggers apoptosis (Beyersmann, 2002; Beyersmann and Haase, 2001; Choi et al., 1988; Duncan et al., 1992; Kim et al., 1999; Walther et al., 2003; Wilhelm et al., 2001). Acute zinc toxicity is rare but has been reported (Duncan et al., 1992) and has been associated with reduced iron absorption and impaired immune function (Whittaker, 1998). Excess zinc may also affect the cellular status of copper and iron (Prasad et al., 1999). How the zinc leads to the improvements in several phenotypes of the mutants and how the flies perceive its potential rescue effects are still open questions. Several mechanisms are feasible. We propose that extra zinc helps to make up for the intrinsic zinc depletion faced by the *parkin* mutants during development, helping them to cope better, most likely by restoring several zinc-dependent functions.

Taken together, these data suggest the importance of metal homeostasis and antioxidant and stress relieving genes induced by MTF-1 in rescuing *parkin* mutant *Drosophila*. Hence, therapeutic approaches that modulate metal bio-availability might also ameliorate the symptoms of Parkinson's disease. This situation may not even be restricted to Parkinson's disease and might inspire research on novel therapeutic approaches to the other neurodegenerative disorders.

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1. Extended lifespan and improved condition of *Drosophila parkin* mutants fed with chelators of redox-active metals.
2. *Parkin* mutant in the fly is largely rescued by metal-responsive transcription factor (MTF-1)
3. Zinc supplement greatly improves the condition of *parkin* mutant *Drosophila.*